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Reduction of Ferric Compounds by Soil Bacteria

BY S. M. BROMFIELD

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SUMMARY: Reduction of ferric lactate by washed cell suspensions of *Bacillus circulans*, *B. megaterium* and *Aerobacter aerogenes* takes place in the presence of suitable hydrogen donors. Ferric hydroxide is reduced when $\alpha\alpha'$-dipyridyl is present in the cell + substrate system. The reduction of ferric compounds by washed cells + substrate is similar to the reduction of methylene blue. Culture fluids of *B. circulans* do not reduce ferric iron in the absence of normal cells.

The reduction of ferric compounds by soil bacteria is well established, although the mechanism by which the reduction is brought about is still not understood. Halvorson & Starkey (1927) concluded that the solution and reduction of iron by bacteria was the result of an increase in hydrogen-ion concentration due to acid formation during the decomposition of organic matter and also to the removal of oxygen by metabolic processes. It was considered that the net effect of these changes was to displace the equilibrium between ferrous and ferric iron towards the formation of ferrous iron. Roberts (1947) attempted to discover how strains of *Bacillus polymyxa* could rapidly reduce ferric hydroxide in glucose media. This was done by studying the dissimilation of glucose under anaerobic conditions; but from the results obtained it was not possible to suggest a mechanism for the reduction. It was found that resting cells of *B. polymyxa* in phosphate buffer and glucose reduced ferric hydroxide, and therefore it was suggested that either a 3-carbon intermediate or formic acid or hydrogen was the hydrogen-donor involved in the reduction. However, it was noted that ferric hydroxide was not reduced when glucose was replaced by formate or hydrogen in resting cell experiments. Bloomfield (1951, 1953) studied iron reduction in relation to gley and podzol formation, and found that sterile solutions of fermented grass and water extracts of Kauri pine leaves and bark reduced ferric iron. He advanced the view that the reduction of ferric ions by bacteria may be only a side reaction of the fermentation. Bétrémieux (1951) suggested that the medium became reducing because bacteria removed molecular oxygen, and that when this had taken place other sources of oxygen such as oxides of iron and manganese were used. The work of Bromfield (1954) indicated that the reduction of iron oxide is not, in fact, due to a bacterial utilization of the oxygen of ferric oxide to metabolize a substrate, as is the case when nitrate and sulphate are reduced by certain micro-organisms. The work described below is a continuation of earlier work (Bromfield, 1954), and was undertaken to correlate the views of various workers and to elucidate the mechanism by which iron compounds are reduced by bacteria.

METHODS

The following media were used:

Medium A. KH_2PO_4, 0·05 g.; $MgSO_4.7H_2O$, 0·02 g.; $(NH_4)_2SO_4$, 0·1 g., sucrose, 0·5 g.; $CaCO_3$, 0·5 g.; Difco yeast extract, 0·015 g.; distilled water, 100 ml.; adjusted to pH 7·0.

Medium B. This medium was identical with medium A except for sucrose, 1·0 g.; Difco yeast extract, 0·3 g.; and it was solidified with 2 % (w/v) agar. Both media were sterilized by autoclaving for 15 min. at 15 lb./sq.in.

Total iron was determined by the method of Piper (1942). Ferrous iron was determined directly by means of $\alpha\alpha'$-dipyridyl on centrifuged samples of the culture fluids.

Washed bacterial cells were prepared from 24 to 48 hr. cultures grown on medium B in Roux bottles at 30°; the period of incubation depended on the rate of growth of the given organism. The cells were suspended in distilled water, centrifuged, resuspended in distilled water and allowed to stand in a shallow layer in flasks at 4° for 18 hr. The suspensions were then centrifuged and the cells resuspended in distilled water. Cells prepared in this way did not completely decolorize methylene blue in 1–2 hr. unless a suitable substrate was added. The washed cells were used as soon as they were prepared as described above.

Reduction of methylene blue and of iron compounds by washed cells was studied in Thunberg tubes, after the method of Quastel, Stephenson & Whetham (1925). The tubes contained 1 ml. 0·01 M-substrate, 2 ml. distilled water, *c.* 10 mg. calcium carbonate and 1 ml. methylene blue solution (1 mg./6 ml. water) or 1 ml. suspension or solution of the required iron compound. The calcium carbonate was used instead of 0·2 M-phosphate buffer because reduction of iron by growing cultures was originally studied in media of high calcium carbonate and low phosphate concentrations. The Thunberg tubes were evacuated for 5 min. at a vacuum of 2–5 mm. Hg, the contents mixed and incubated in a water-bath at 40°; the time necessary for reduction to occur was then noted.

RESULTS

Solution and reduction of iron compounds by culture fluids of Bacillus circulans

To determine whether *Bacillus circulans* reduced iron by forming reducing substances which accumulated in the culture, the following experiment was carried out: 25 ml. vol. of medium A in 6 × 1 in. test-tubes were inoculated with *B. circulans* and incubated aerobically at 30° for 5 days; the cultures were then either autoclaved, treated with 1 ml. chloroform, centrifuged or left untreated. To these cultures 1 ml. of 1 % (w/v) sterile ferric hydroxide suspension or 1 % (w/v) sterile ferric chloride solution was added and the cultures incubated anaerobically for 24 hr. at 30°. Determinations of total iron in solution, ferrous iron in solution and pH value were made on the cultures. The results are given in Table 1 which show that the constituents of the cultures possessed virtually no capacity for iron reduction or iron solution unless untreated bacterial cells were present.

Reduction of iron compounds by suspensions of washed bacterial cells

The first step was to determine some of the dehydrogenase systems present in cells of *Bacillus circulans*. The substrates tested and the times for complete reduction of methylene blue are given in Table 2, which shows that the washed cells contained active malic and glucose dehydrogenase, weak succinic and lactic dehydrogenase and no formic dehydrogenase.

Table 1. *Solution and reduction of ferric compounds by 5-day cultures of* Bacillus circulans *after various treatments under anaerobic conditions. Determinations 24 hr. after the addition of iron compound*

	Iron compound added				
	$FeCl_3$	$Fe(OH)_3$	$FeCl_3$	$Fe(OH)_3$	$Fe(OH)_3$
Treatment given to 5-day culture	pH value		Ferrous iron in solution (mg. Fe^{++}/l.)		Total iron in solution* (mg./l.)
Autoclaved	5·6	6·6	1·5	0	0
Centrifuged	4·7	6·4	0·5	1·5	2
1 ml. chloroform	6·5	6·5	0	2	2
Untreated	6·3	6·2	20	25	27

* Total iron only determined in tubes which received $Fe(OH)_3$.

Table 2. *Time for complete reduction of methylene blue by washed cells of* Bacillus circulans *in presence of various substrates*

Substrate (0·01 M)	Time for complete reduction (min.)
Glucose	9
Succinate	30
Malate	5
Lactate	50
Formate	60
Water	60

An attempt was made to reduce ferric hydroxide by washed cells in the presence of the substrates listed in Table 2. The Thunberg tubes and their contents were incubated for 1 hr. and then tested for ferrous iron with a 0·1 % solution of $\alpha\alpha'$-dipyridyl in 0·01 N-HCl. A very weak positive result for ferrous iron was given in those tubes which contained glucose. It was observed in other tests that when $\alpha\alpha'$-dipyridyl was added initially, ferrous iron was detected quite readily in Thunberg tubes which contained ferric hydroxide; and $\alpha\alpha'$-dipyridyl did not affect the time needed for methylene blue reduction. By using $\alpha\alpha'$-dipyridyl in the iron-reducing system it was thus possible to compare the time for the appearance of ferrous dipyridyl with the time for complete reduction of methylene blue. The results of this comparison are given in Table 3. The times taken for the appearance of ferrous dipyridyl and for the complete reduction of methylene blue were correlated. The ferrous dipyridyl, however, appeared before the methylene blue was completely reduced. The intensity of the colour of ferrous dipyridyl in the various substrates increased with time; after 2 hr. it was inversely related to the time needed for complete reduction of methylene blue.

Table 3. *Times for complete reduction of methylene blue and appearance of ferrous dipyridyl with washed cells of* Bacillus circulans *in various substrates*

Substrate (0·01 M)	Time for methylene blue reduction (min.)	Time for appearance of ferrous dipyridyl (min.)
Glucose	14	13
Succinate	50	19
Malate	8	8
Lactate	99	80
Formate	>120	90
Water	>120	90

Table 4. *Effect of various enzyme inhibitors on the time for methylene blue reduction and on the formation of ferrous dipyridyl in a washed cell + malate system*

Organism: *Bacillus circulans.*

Treatment	Time for complete reduction of methylene blue (min.)	Appearance of ferrous dipyridyl
1 ml. 1 % KCN	10	+
1 ml. 1 % NaN_3	15	+
1 ml. chloroform	>200	−
1 ml. toluene	>200	−
Ethanol treated cells	70	+
Boiled cells	>200	−
No treatment	14	+
No malate	>120	−

Effect of enzyme inhibitors on reduction

The effect of various enzyme inhibitors and of heat on the reduction of ferric hydroxide and methylene blue by washed cells of *Bacillus circulans* in the presence of 0·01 M-sodium malate was studied. The inhibitors used were: 1 % (w/v) KCN (giving a final concentration of 0·17 %); 1 % (w/v) sodium azide; chloroform; toluene (these four substances were added in 1 ml. volumes directly to the rest of the solutions in the Thunberg tubes); 50 % (v/v) aqueous ethanol; the cells were treated for 30 min. and then 1 ml. of ethanol-treated cells added to the Thunberg tubes. The heat treatment consisted of boiling the cell suspension for 2 min.; then 1 ml. suspension was added to the tubes. The effect of these treatments is given in Table 4. The dehydrogenase inhibitors and boiling prevented iron reduction, whereas the poisons which did not inhibit or only partially inhibited the dehydrogenase system did not prevent iron reduction.

Reduction of ferric lactate by washed cells of Bacillus circulans

Ferric lactate prepared from 10 ml. 1 % (w/v) ferric chloride and 10 ml. of 10 % (v/v) lactic acid and neutralized with calcium carbonate to pH 5·8 was used as the iron source in Thunberg tube experiments. The solution was used as soon as prepared; it did not give a reaction for ferrous iron with $\alpha\alpha'$-dipyridyl. The treatments and results are set out in Table 5. The colour of ferrous dipyridyl was quite strong in tubes which contained fresh cells, malate or glucose, but very weak in the tubes without substrate and in those which received boiled cell suspension or no cells at all. Good reaction for ferrous iron was given immediately when $\alpha\alpha'$-dipyridyl solution was added to tubes containing substrate + ferric lactate + fresh cells which had been incubated for as little as 10 min.

Table 5. *Reduction of ferric lactate by washed cells of* Bacillus circulans

	Reaction for ferrous iron					
	$\alpha\alpha'$-Dipyridyl added initially			$\alpha\alpha'$-Dipyridyl added after 10 min. incubation		
Substrate (0·01 M)	Fresh cells	Boiled cells	No. cells	Fresh cells	Boiled cells	No. cells
Glucose	++++	–	–	++++	–	–
Malate	++++	–	–	++++	–	–
Water	+	–	–	+	–	–

Effect of ferric hydroxide, ferric lactate and sodium lactate on methylene blue reduction

To determine the effect of ferric hydroxide, ferric lactate and sodium lactate on the time for complete reduction of methylene blue by malic dehydrogenase, Thunberg tubes were set up as follows. Each tube contained malate + cells + calcium carbonate and either 1 ml. 1 % (w/v) ferric hydroxide suspension, or 1 ml. ferric lactate solution (see above), or 1 ml. 5 % (w/v) sodium lactate or 1 ml. water. The times for complete reduction were 5, 60, 5 and 5 min. respectively. At the end of this experiment the various tubes were tested for ferrous iron; only those which had received ferric lactate gave a positive reaction. These results indicate that lactate and ferric hydroxide have no effect on the time for complete reduction of methylene blue by malic dehydrogenase, whilst soluble ferric iron considerably delays the reduction time.

Reduction of iron compounds by washed cells of Bacillus megaterium *and* Aerobacter aerogenes

It was found previously (Bromfield, 1954) that growing cultures of *Bacillus megaterium* and *Aerobacter aerogenes* were, respectively, poor and fairly good reducers of ferric hydroxide in medium A when compared with *B. circulans*. Experiments similar to some of those described above for the washed cells of *B. circulans* were carried out with cells of these other two bacteria. It was found that their cells, in the presence of a suitable dehydrogenase substrate, behaved similarly to cells of *B. circulans*.

DISCUSSION

The mechanisms for the reduction of ferric compounds by bacteria and by sterile plant extracts differ (Bloomfield, 1951, 1953) since the culture fluid of *Bacillus circulans* does not reduce these compounds unless untreated cells are present (Table 1) whereas sterile plant extracts do. The reduction of ferric iron by the bacteria examined appears to be closely associated with dehydrogenase activity for the following reasons: (*a*) the time for methylene blue reduction is comparable to the time for the appearance of ferrous iron in the presence of an active dehydrogenase (Table 3); (*b*) the dehydrogenase inhibiting treatments (Table 4) which prevent methylene blue reduction also prevent iron reduction; (*c*) soluble ferric iron increases the time required for complete reduction of methylene blue when the two are mixed.

An active dehydrogenase system, however, is not enough in itself to cause reduction of ferric hydroxide, since ferric iron is only reduced by such a system when the iron is in solution or when there is a complex-forming substance such as $\alpha\alpha'$-dipyridyl present. These relations may explain why some growing cultures of bacteria reduce ferric hydroxide more than others (Bromfield, 1954). These differences may be due to the production of variable amounts of specific organic acids or complex-forming substances which affect the solubility of ferric hydroxide. If this be so then iron reduction should occur when substances are formed which can increase the solubility of ferric hydroxide without interfering with the maintenance of active dehydrogenase systems.

A mixed culture of bacteria might reduce ferric hydroxide more readily than a pure culture of any one organism of the mixture. Bacterial reduction may be important in increasing the nutrient availability of iron and manganese oxides and also in determining the movement of iron and manganese in soil profiles, especially where there is abundant available organic matter.

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Tryptophan Metabolism and its relation to Phage Resistance in *Escherichia coli*

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SUMMARY: A series of phage-susceptible tryptophan-requiring mutants of *Escherichia coli*, strain B, were found to fall into five distinct phenotypes as determined by their alternate growth response to anthranilic acid and indole, and by the substances which they accumulated in the culture fluids. An unidentified substance partially characterized by colorimetric reactions and absorption spectrum accumulated in the culture fluid of two of these types. Tryptophan-requiring T_1 phage resistant mutants (*B/1, trp*) which were readily obtained by phage selection could not be isolated by the penicillin method. This mutant represents a unique tryptophanless phenotype, not only in terms of its resistance to T_1 phage, but also on the basis of: (1) accumulation of a new metabolic product; (2) growth stimulation by histidine and tyrosine; (3) suppression of accumulation of certain metabolic products by the other tryptophan auxotrophs; (4) epistatic expression of phenotype when superimposed on the tryptophan auxotrophs by secondary mutation.

In analysing mutants of *Escherichia coli*, strain B, which are resistant to the T series of coli phages a consistent and reproducible association is found between resistance to the T_1 coli phage and a growth requirement for tryptophan (Anderson, 1946). It was felt that an elucidation of the nature of this metabolic lesion might lead to some insight into the mechanism of phage resistance and, by converse analogy, phage susceptibility. In order to determine the nature of the tryptophan requirement of this mutant, we used a method of comparative analysis which utilizes a series of tryptophan auxotrophs derived from the same parent strain by the penicillin selection method (Davis, 1949). The pattern of the tryptophan-deficient phenotypes so obtained involved the same series of genetic blocks in the synthesis of anthranilic acid and its conversion to tryptophan via indole as previously found in mutants of Neurospora (Tatum, Bonner & Beadle, 1943) and other *E. coli* strains (Davis, 1951). In addition to these previously well-described types of mutants, several new types have been found which are characterized by the excretion of an abnormal, and as yet unidentified, metabolic product.

It was originally hoped that one of the tryptophan auxotrophs would show a resistance to phage T_1, thus establishing an unequivocal relationship between the resistance and nutritional deficiency. Repeated isolations, however, have revealed no tryptophan auxotrophs which are resistant to T_1 or any of the other coli phages. The tryptophan-requiring mutant which is resistant to T_1 can only be isolated by means of selection for resistance by the action of T_1 phage. As will be developed here, this organism represents an independent and unique phenotype, not only in terms of its resistance to phage, but also in terms of its tryptophan requirement.

MATERIAL AND METHODS

Basal medium. The composition of the defined inorganic salts + glucose medium has been previously described (Gots & Chu, 1952).

Tryptophan auxotrophs. Mutants of *Escherichia coli*, strain B, which were unable to grow in the basal medium unless tryptophan was supplied, were obtained by ultraviolet irradiation followed by selection with penicillin (Davis, 1949). These mutants will henceforth be referred to as the 'tryptophan auxotrophs'.

Phage-resistant mutants. Mutants resistant to the action of the T series of coli phages were obtained by plating approximately 2×10^8 bacteria with sufficient phage to yield confluent lysis. The nomenclature of Demerec & Fano (1945) will be used to describe these mutants. For example, *B/1, 5* refers to that mutant of *Escherichia coli*, strain B, which survived lysis by T_1 and was subsequently found to be also resistant to T_5.

Accumulations. Cultures were obtained by the growth of the tryptophan-requiring mutants in a suboptimal (2–5 μg./ml.) concentration of tryptophan for at least 48 hr. The bacteria were removed by centrifugation with and without the prior addition of trichloroacetic acid. The detection of accumulated metabolic products in the culture fluid supernatants was accomplished by a variety of methods. Colorimetric analyses were performed with a number of tests for indole and derivatives (Fellers & Clough, 1925; Gordon & Weber, 1951), anthranilic acid and derivatives (Kikkawa, 1941), and diazotizable amines (Bratton & Marshall, 1939). The latter proved the most versatile. It was applied for the quantitative measurement of both anthranilic acid and indole (Eckert, 1943). Separation of the accumulated substances was facilitated by ascending paper chromatography using a *n*-butanol + acetic acid + water (4:1:1) solvent mixture. Ultraviolet spectra were measured with a Beckman quartz spectrophotometer (model DU) and fluorescence was measured with a Klett-Summerson fluorimeter. Syntrophism was detected by auxanographic techniques.

EXPERIMENTAL

Tryptophan auxotrophs

A total of thirty-four independent isolations of tryptophan-requiring mutants of *Escherichia coli*, strain B, could all be classified into three nutritional phenotypes according to the pattern of alternate requirements supplied by anthranilic acid or indole. Type *trp-1* (e.g. strain B-106) responded to anthranilic acid or indole, indicating a block in the synthesis of anthranilic acid; *trp-2* (e.g. B-37) used indole but not anthranilic acid, indicating a block in the conversion of anthranilic acid to indole; and type *trp-3* (e.g. B-82) responded only to tryptophan, indicating an impairment in the conversion of indole to tryptophan. The following substances were unable to replace tryptophan as a growth requirement for any of the organisms: casein acid hydrolysate, B vitamins, cinnamic acid, indole-3-acetic acid, indole-3-butyric acid, skatole, quinolinic acid, kynurenine,* kynurenic acid, 3-OH-anthranilic acid* and tryptamine.

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* We are indebted to Dr D. Bonner for a supply of these compounds.

In addition to these types of organisms, mutants with a triple requirement for tryptophan, tyrosine and phenylalanine were also found. These represent an early block in the synthesis of a common aromatic precursor (Davis, 1951). Anthranilic acid or indole could replace the tryptophan part of the multiple requirement.

In order to establish the exact nature of the genetic blocks, as well as to determine non-allelism within a group of similar nutritional types, the culture fluids of the auxotrophs were examined for accumulated intermediates. By the methods employed it was shown that some members of *trp-2* accumulated anthranilic acid and some members of *trp-3* accumulated indole in relatively high concentrations, but no evidence for any accumulated substance could be found with the *trp-1* organisms.

Two additional phenotypes were found, *trp-2a* (e.g. strain B-42) and *trp-3a* (e.g. B-81) which differ from the other members of their respective nutritional groups in that they accumulate neither anthranilic acid nor indole, but accumulate an unidentified indole-like substance. This substance appears to be the same in the two types which produce it. It is readily characterized by its tryptophan-like ultraviolet absorption spectrum with a maximum absorption at 280 $m\mu$. It gives a blue chromogen with the Bratton & Marshall (1939) test, a positive xanthydrol reaction, and a red colour with the ferric chloride test for indole acetic acid (Gordon & Weber, 1951). Since it is unable to feed the *trp-1* mutants its role as a normal intermediate cannot readily be assessed. Details of chemical analyses, isolation and characterization will be presented in a future publication.

Mutants resistant to T_1 coli phage

The types of phage-resistant mutants of *Escherichia coli*, strain B, have been thoroughly described by Demerec & Fano (1945), Beale (1948) and Bryson & Davidson (1951). The majority of the T_1 resistant mutants were also resistant to T_5 coli phage (*B/1, 5*) and were like the wild type nutritionally. About 10–25 % of the total T_1 resistant population were susceptible to T_5 and manifested a requirement for tryptophan (*B/1, trp*). An occasional, but rare, *B/1* not requiring tryptophan was also found. As has been reported by Bryson & Davidson (1951), we have also demonstrated a tryptophan-requirement among some of the mutants which are resistant to both T_1 and T_5 (*B/1, 5, trp*). These, however, probably represented two-step mutations (*B/1, trp/1h, 5*) involving secondary resistance to T_{1h}, the host range mutant of T_1 which is present in T_1 stocks and mimics T_5 in its host range activity. This was supported by the fact that a tryptophan-requirement was never found among the *B/5, 1* mutants isolated by the selective action of T_5 which is devoid of T_{1h}. Also, when the action of T_{1h} was prevented by citrate (unpublished observation), no tryptophan requirement could be found in any of the *B/1, 5* mutants isolated by the action of T_1. Numerous complexities have been revealed in these analyses which, not being important to our present discussion, will be reserved for a future presentation.

All of the *B/1, trp* mutants so far examined fell into nutritional type *trp-3* in that neither indole nor anthranilic acid could replace tryptophan for growth. They differed, however, from the previously described members of this group in that neither indole nor the '280' substance was accumulated. With the ordinary chemical tests employed, no difference, with respect to accumulation, could be shown between the parent wild type and the *B/1, trp* mutants. However, by spectrophotometric analysis a weak, but distinct, absorption was evident with a peak at 338 mμ. This is shown in Fig. 1. Attempts to increase the concentration of this material by addition of casein hydrolysate, yeast extract or B vitamins were unsuccessful. Its absorption pattern is reminiscent of the absorption of the reduced pyridine nucleus as given, e.g. by reduced diphosphopyridine nucleotide (DPN). That this was not reduced DPN was shown by the use of a bacterial DPN-cytochrome-reductase (Brodie, 1952). The flavoprotein, which rapidly oxidizes reduced DPN with loss of 340 mμ. absorption, had no effect on the absorption of the *B/1, trp* supernatant.

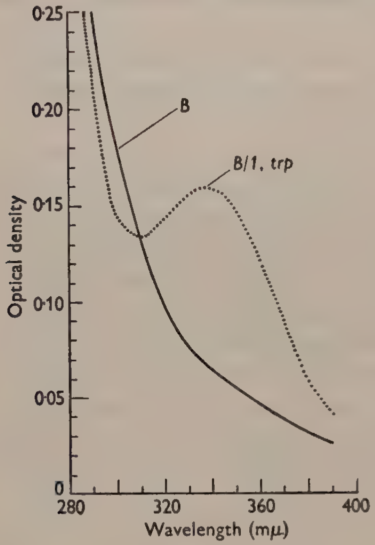

Fig. 1

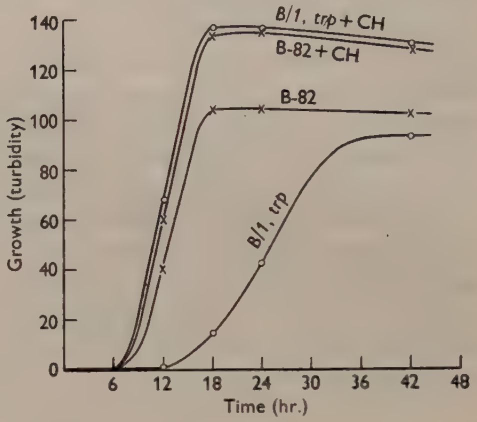

Fig. 2

Fig. 1. Ultraviolet absorption spectra of culture supernatants of *Escherichia coli*, strains B and *B/1, trp*. Supernatants were obtained from 48 hr. cultures grown in minimal medium containing 5 μg. tryptophan/ml.

Fig. 2. Comparison of growth rates of *B/1, trp* and a *trp-3* type (B-82). Media consisted of minimal medium containing 10 μg. tryptophan/ml. with and without casein hydrolysate (CH), 0·1 %.

Growth response to tryptophan

A striking difference in the type of tryptophan requirement between *B/1, trp* and the other auxotrophic mutants was seen in the nature of the dose-response curves. It can be seen from Fig. 2 that the response of *B/1, trp* to tryptophan was slower in rate and lower in maximum growth obtained when compared with one of the tryptophan-requiring phage susceptible mutants. All of the latter were similar in this respect. This difference shown by the *B/1, trp* mutant was completely nullified in the presence of an amino acid environment such as casein hydrolysate. Similar amino acid effects have been noted by Anderson (1946) and Raff & Cohen (1950). An analysis of the separate amino acids contained in casein hydrolysate revealed that the entire effect of the latter could be replaced by histidine + tyrosine. As is shown in Fig. 3, histidine acts by increasing the growth rate in a given concentration of tryptophan and the presence of tyrosine permits an elevation of the maximum growth obtained. The mechanism of these stimulations is unknown.

Phage-resistant mutants of the tryptophan auxotrophs

When the phage-sensitive tryptophan auxotrophs were subjected to the action of T_1 and T_5 coli phages a series of phage-resistant mutants were obtained which, in terms of their phage-resistance patterns, were the same as those obtained by the action of the phages on the wild type. These were examined for alterations of the original phenotype as measured by nutritional responses and accumulations. Those mutants which were resistant to both T_1 and T_5 (*trp/1, 5* or *trp/5, 1*) were indistinguishable from the phenotype of the sensitive parent from which they were derived. However, those which were resistant to T_1 but not T_5 (*trp/1*) were no longer phenotypically identical with their respective parents. In all cases, the *trp/1* mutant could no longer respond to any of the tryptophan precursors, thus acting as a *trp-3* nutritional type even though its original parent may have been a *trp-1* or *trp-2* type. Furthermore, those organisms which showed accumulations could no longer accumulate these substances when carrying the superimposed mutation to T_1 resistance. Thus, a condition of epistasis was evident in that the *trp/1* mutants exhibited a phenotype indistinguishable from that of *B/1, trp* regardless of what the original parent phenotype may have been. A summary of these data is given in Table 1. The *B/1* phage-resistant type shown in the table is a *B/1, trp* mutant. Though *B/1* which do not require tryptophan have been found, these have not been included in the table since their occurrence is rare and no counterpart has been found among the phage-resistant derivatives of the tryptophan auxotrophs.

The alteration of the nutritional phenotype by the mutation to T_1 resistance is understandable on the basis of the inability of the original *B/1, trp* mutant to respond to the intermediates preceding tryptophan. However, the suppression of the accumulation of the intermediates cannot be explained so readily on the basis of a double genetic block. In reconstruction experiments where an indole accumulator (B-82) and *B/1, trp* were simultaneously inoculated into the minimal medium containing a suboptimal concentration of tryptophan, it was found that the accumulation of indole in the culture fluid was greatly suppressed. This indicated that the *B/1, trp* organism could prevent the accumulation of indole by strain B-82. More rigorous proof was obtained by the incorporation of sterile culture fluids of *B/1, trp* into medium inoculated with B-82. Fig. 4 shows that such addition to the media markedly depressed the accumulation of indole as compared with controls in which similar

Table 1. *Phenotypes of* Escherichia coli *involving tryptophan requirement and phage resistance*

Strain	Phage-resistant phenotype	Phage pattern			Growth response				Accumulation of intermediates
		T_1	T_{1b}	T_5	O	A	I	T	
B	Wild type	S	S	S	+	+	+	+	None
	B/1, 5	R	R	R	+	+	+	+	None
	B/1	R	S	S	−	−	−	+	None
B-106	*trp-1*	S	S	S	−	+	+	+	None
	trp-1/1, 5	R	R	R	−	+	+	+	None
	trp-1/1	R	S	S	−	−	−	+	None
B-37	*trp-2*	S	S	S	−	−	+	+	Anthranilic acid
	trp-2/1, 5	R	R	R	−	−	+	+	Anthranilic acid
	trp-2/1	R	S	S	−	−	−	+	None
B-42	*trp-2a*	S	S	S	−	−	+	+	'280' substance
	trp-2a/1, 5	R	R	R	−	−	+	+	'280' substance
	trp-2a/1	R	S	S	−	−	−	+	None
B-82	*trp-3*	S	S	S	−	−	−	+	Indole
	trp-3/1, 5	R	R	R	−	−	−	+	Indole
	trp-3/1	R	S	S	−	−	−	+	None
B-81	*trp-3a*	S	S	S	−	−	−	+	'280' substance
	trp-3a/1, 5	R	R	R	−	−	−	+	'280' substance
	trp-3a/1	R	S	S	−	−	−	+	None

S = susceptible and R = resistant to phage.
O = minimal media; A = minimal plus anthranilic acid; I = plus indole; T = plus tryptophan.

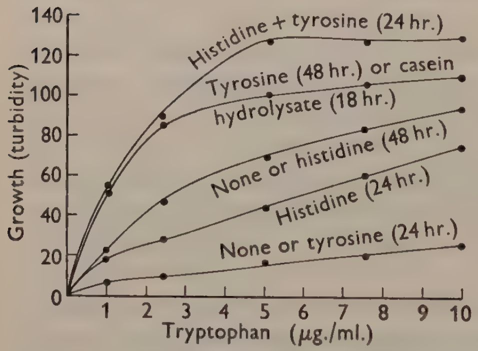

Fig. 3

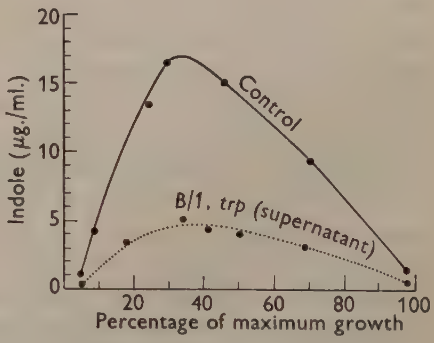

Fig. 4

Fig. 3. Tryptophan dose-response curves of *B/1, trp*. Curves are shown for various incubation periods with and without (none) tyrosine (10 μg./ml.), histidine (10 μg./ml.) and casein hydrolysate (0·1 %).

Fig. 4. Indole accumulation by strain B-82. Double strength minimal medium was diluted 1:1 with sterile filtrates from 48 hr. cultures of (*a*) wild type grown in minimal medium (control), and (*b*) *B/1, trp* grown in minimal medium + tryptophan (5 μg./ml.). These were supplemented with tryptophan in the following concentrations: 0·0, 0·1, 0·25, 0·5, 1·0, 2·5, 5·0 and 10·0 μg./ml. After inoculation with strain B-82, percentage of maximal growth was determined from dose-response curves after 48 hr. incubation. Indole concentration was measured in supernatants of the 48 hr. cultures.

treatment was made with culture fluids obtained from the wild type. The curves shown were obtained by determining indole concentration in a series of cultures containing increasing amounts of tryptophan. Indole accumulation by this mutant is linear with growth response up to a maximum after which increased growth obtained with increase in tryptophan content results in a suppression of indole accumulation. Since residual tryptophan was present in the supernatants of the *B/1, trp* cultures, its addition would be expected to alter indole accumulation by virtue of the alteration of growth response. It was thus necessary to correlate indole accumulation with percentage of maximum growth as obtained from a tryptophan dose-response curve. With this correction for alteration of growth response it is obvious, as shown in the figure, that indole accumulation by B-82 was markedly suppressed when supernatants from *B/1, trp* were present in the medium. The abscissal displacement of the points shown represent the growth stimulation brought about by the presence of the *B/1, trp* supernatant. Thus, where 1 μg. of tryptophan per ml. (4th point) allowed 30 % of maximal growth, this was increased to 41 % in the presence of the *B/1, trp* supernatant. Similar experiments with strain B-37 showed that anthranilic acid accumulation is also, but not as markedly, inhibited by *B/1, trp* filtrates.

DISCUSSION

The original object of this study was to identify the nature of the tryptophan requirement of the T_1 resistant mutant by comparison with a series of comparatively well-defined auxotrophs, representing genetic blocks in the sequence of tryptophan synthesis from anthranilic acid. No identity could be established. Instead, the *B/1, trp* mutant was found to be a different tryptophanless phenotype. This was shown by its unique type of accumulation, its growth stimulation by histidine and tyrosine, and its epistatic behaviour when present with other tryptophanless mutations.

It was particularly disturbing to find that though tryptophanless auxotrophs are isolated readily from a population of *Escherichia coli* they are never found to be phage resistant and always fall into one of five readily classified phenotypes. Repeated attempts to isolate the *B/1, trp* type by selection for its tryptophan-requiring character have failed. This was true even with populations which were known to be heavily concentrated with respect to *B/1, trp* as determined by fluctuation analysis and selection for phage resistance.

It is possible that the tryptophan requirement of *B/1, trp* is not due to a deficiency in tryptophan synthesis but may be an expression of a deleterious or growth-limiting reaction which can be neutralized by tryptophan. Examination of the effects of culture filtrates and sonically prepared extracts of *B/1, trp* on the growth of the wild type, the tryptophan auxotrophs and the *B/1, 5* mutant revealed no inhibitory action. Also, the presence of these fractions did not affect the course of T_1 production by the wild type. If an inhibitory mechanism is responsible for the tryptophan requirement of *B/1, trp*, it is of a nature too subtle to permit experimental detection.

Anderson (1944) has presented a generalized scheme involving dichotomous interrelationships between phage resistances and metabolic changes leading to increased growth requirement. From the data presented here we have formulated an extension of the scheme only as a tentative representation to allow for integration of the observed series of phenotypes. Its strength rests on the principles of biochemical genetics which allow for a correlation of phenotypic expressions with postulated genetic breaks.

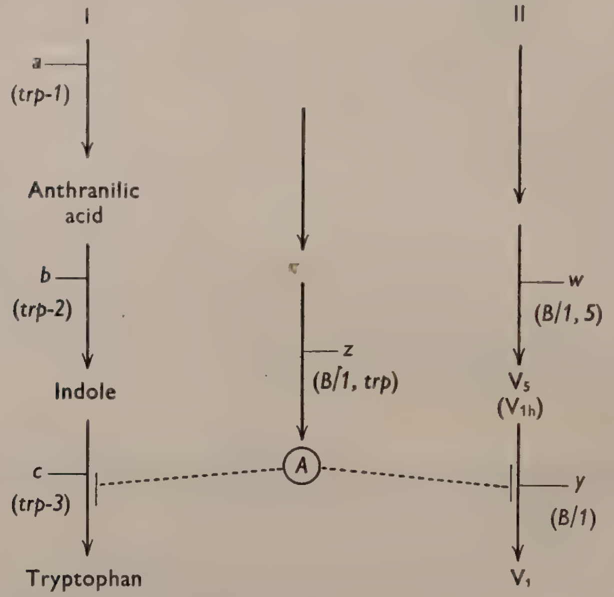

Fig. 5. Tentative scheme depicting mutational sites of observed phenotypes.

Fig. 5 shows two independent pathways, I and II. Pathway I leads to the formation of tryptophan via anthranilic acid and indole. Phage-sensitive tryptophan auxotrophs would result from mutations at sites *a*, *b* and *c* yielding *trp-1*, *trp-2* and *trp-3* types respectively. Location of *trp-2a* and *trp-3a* cannot be made accurately until the nature of the substance which they accumulate is determined.

The other pathway (II) leads to the formation of the factors which must be present to enable the bacterial cell to react with the phages T_1 and T_5. Using Anderson's nomenclature (1944), these are referred to as V_1 and V_5 respectively. The lack of these factors would render the bacteria resistant to the phages. A mutation at site *w* would yield the *B/1, 5* or *B/5, 1* types, and a mutation at site *y* would result in the *B/1* type. These would all be prototrophic. The V_5 factor has been depicted as an incomplete V_1 in order to allow for the fact that no *B/5* mutant, to the best of our knowledge, has yet been described. Since the host range of T_5 and T_{1h} are identical, V_5 and V_{1h} are either the same or very closely related.

This concept is also in accord with the findings of Garen & Puck (1951) who have examined the first two steps of the invasion of *Escherichia coli*, strain B, and its T_1 resistant mutants by the T_1 phage. The *B/1, 5* mutant was resistant to T_1 because of the failure of the primary reversible attachment to occur. This step could be controlled by V_5. The *B/1* mutant was able to carry out this reversible attachment (V_5 present), but was unable to permit subsequent irreversible transformation (V_1 absent).

In order to explain the *B/1, trp* mutation it is necessary to link the tryptophan pathway with the V_1 pathway. This can be done by a common factor, '*A*', which places both pathways under unit control. The presence or absence of this factor would be controlled at site *z*. If in the normal cell, *A* is required both for formation of tryptophan from indole and for completion of V_1, a 'loss' mutation at *z* would result in the observed phenotype. However, the same phenotype could be obtained by a 'gain' mutation at *z* in which case the normally absent *A* would exercise a suppressive mechanism at the sites indicated. The data available at present cannot definitely distinguish between these two possibilities. In order to explain the stimulatory effects of histidine and tyrosine, as well as the suppression of accumulations, it is also necessary to postulate that a mutation involving *z* must lead to other metabolic disturbances and imbalances.

The mutant which is resistant to both T_1 and T_5 and exhibits a tryptophan requirement cannot be fitted into the postulated scheme as a single mutation. As stated before this probably represents a two-step mutation obtained by secondary selection with T_{1h}. A mutation at *z* followed by another at *w* would give this phenotype.

It must be emphasized that the exposition presented above applies only to the tryptophan deficiency found among spontaneously occurring mutants. Bryson & Davidson (1951), by means of ultraviolet induction, obtained phage-resistant mutants of *Escherichia coli*, strain *B/r*, which exhibited a variety of nutritional requirements. By back mutation analysis involving reversions to prototrophy, many of these have been shown to be multiple phenotypes or double mutants.

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Enzymes concerned with Gas Formation by some Coliform Bacteria

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SUMMARY: The absence of gas produced by certain coliform organisms during growth at elevated temperatures is not a result of an interference with the enzymes concerned, but rather an interference with the mechanism which brings about the appearance of these adaptive enzymes. A reconsideration of some of the evidence tends to support the view that formic hydrogenlyase is a separate entity, distinct from hydrogenase and formic dehydrogenase.

In the course of a study of coliform bacteria from soil, strains were encountered which had the property of fermenting glucose with production of acid and gas at 30° (their optimum temperature for growth) and of producing acid but no gas at 37°. This behaviour is obviously similar to that of non-faecal strains of *Escherichia coli*, whose optimum temperature for acid and gas production is 37°, and which give acid but no gas from lactose at 44°, a fact of some importance in water analysis. Coliform organisms incapable of producing gas at higher temperatures have been described by several workers (e.g. Allen & Harrison, 1936; Hiscox, 1934; Oxley, 1938) and have been referred to as pseudo-microaerogenic coliforms (Prescott, Winslow & McCrady, 1946). No explanation of the phenomenon in enzymological terms has been given.

When these phenomena are observed for diagnostic purposes, the organisms are grown in a vessel containing sugar peptone water with a Durham tube, and the results are not quantitative. The more quantitative methods of assay of the factors concerned, described below, might make it possible to decide whether failure to produce gas at 37° (soil origin) or 44° (water origin) is due to the thermolability of the enzyme system concerned, or to failure of the organisms to elaborate these enzyme systems (or some part of them) at these higher temperatures.

METHODS

Organisms. Two strains of coliform organisms, B6 and C59, were used; their biochemical properties are given in Table 1. It is of some interest that at 44° strain C59 not only produces no gas, but fails to utilize citrate when this is the sole source of carbon.

Preparation of cell suspensions. The organisms were grown in peptone broth to which glucose (1 %, w/v) and 10 μmole $FeCl_3$/l. were added immediately before inoculation. Strain B6 was incubated at 30°, strain C59 at 37° for 16 hr. The cultures (usually 1 l.) were cooled rapidly to 5–8° in iced water and centrifuged at 2500 r.p.m. for 20 min. The cells were washed twice with cool 0·01 M-phosphate buffer (pH 7·0) and finally resuspended in 25 ml. of the same phosphate solution. The dry weight of bacteria in the suspensions was of the order of 2 mg./ml., as measured by protein estimation by the method of Stickland (1951).

Table 1. *Biochemical reactions of coliform organisms* B6 *and* C59 *at different growth temperatures*

Organism:	B6		C59		
	Growth temperature				
	30°	37°	30°	37°	44°
	Reactions				
Indole	−	−	−	−	−
Methyl red (MR)	+	+	−	+	+
Acetoin	−	−	+	(*)	−
Citrate	−	−	+	+	−
Glucose	A and G	A	A and G	A and G	A
Gelatine	−	−	−	−	−

(*) = a faintly weak positive reaction; A = acid production; G = gas production.

Methods of enzyme assay

Formic dehydrogenase. The usual Thunberg tube procedure was used, with 0·5 ml. phosphate buffer (0·1 M, pH 7·0), 0·5 ml. methylene-blue solution (0·002 M), 0·5 ml. sodium formate (0·1 M) and 0·5 ml. of suitably diluted bacterial suspension in each tube. The evacuated tubes were incubated in a water-bath at the appropriate temperature and the time to 90 % reduction of the methylene blue noted. Blanks with the formate omitted were carried out on every occasion.

Hydrogenase. The test was similar to that for formic dehydrogenase, but the formate was omitted and the tube, after evacuation, filled with oxygen-free H_2, re-evacuated, refilled, and finally left partially evacuated, with *c.* 60 cm. Hg pressure of H_2. Before immersion in the water-bath the tubes were shaken to ensure solution of sufficient H_2 in the solutions.

Formic hydrogenlyase. The usual Warburg manometric technique was used. The vessels contained 1 ml. phosphate buffer (0·1 M, pH 7·0), 1 ml. sodium formate (0·25 M) and 1 ml. bacterial suspension. Vessels were evacuated 3 times and filled with oxygen-free nitrogen, and allowed to equilibrate 10 min. before readings were started; CO_2 was absorbed by 5 % KOH on filter-paper.

Glucose hydrogenlyase. This is a convenient name for the enzyme system which results in hydrogen production when glucose is the substrate. The measurements were made in exactly the same way as those for formic hydrogenlyase except that 1 ml. of 0·25 M-glucose replaced the formate solution, and the phosphate buffer was at pH 6·2 instead of 7·0.

Units. The activity of enzyme is expressed as $Q_{H_2} = \mu$l. H_2 evolved/hr./mg. dry-wt. bacteria. Q_{MB} is a similar expression, in which 1 mole of methylene blue is taken as occupying 22·4 l.

RESULTS

To save repetition, 37° is called the 'higher' temperature for strain B6 and 44° the 'higher' temperature for strain C59. The corresponding normal temperatures are: B6, 30°; C59, 37°.

Absence of formic hydrogenlyase and hydrogenase from suspensions grown at higher temperatures

Both strains grown at their higher temperatures showed complete absence of formic hydrogenlyase and of hydrogenase, and greatly decreased formic dehydrogenase activity compared with those of cultures grown at their normal temperatures (Table 2).

Table 2. *Distribution of formic hydrogenlyase (FHL), hydrogenase (H) and formic dehydrogenase (FD), in suspensions grown at normal and at higher temperatures*

Enzyme activity measured as Q_{H_2} or Q_{MB}

	Enzyme					
	FD Q_{MB}	H Q_{MB}	FHL Q_{H_2}	FD Q_{MB}	H Q_{MB}	FHL Q_{H_2}
Organisms	Normal temperature (30°)			Higher temperature (37°)		
B6	60	16	80	—	—	0
	29	13	77	8	0	0
	17	14	93	13	0	0
	Normal temperature (37°)			Higher temperature (44°)		
C59	88	193	350	11	0	0
	63	47	450	5	0	0
	77	61	149	—	—	0

Stability at the higher temperature of hydrogenlyase in strains B6 *and* C59 *grown at the normal temperature*

When B6 was grown in glucose broth at 30° or C59 in the same medium at 37° the washed suspensions showed, at the normal temperature for each strain, typical glucose hydrogenlyase and formic hydrogenlyase activity with Q_{H_2} values in the range 100–400. When these manometric tests were carried out at the higher temperature for the given strain, the activity was initially as great as, or greater than, that at the normal temperature. In some experiments, however, the rate of H_2 evolution at the higher temperature began to diminish rapidly after about 1 hr. This might indicate a greater instability of the enzymes at the higher than at the normal temperature, and thus might account for the absence of the enzymes in the bacteria grown at the higher temperature. However, it was soon found that the cessation of H_2 evolution was to be attributed not specifically to the higher temperature, but to the low pH value caused by glucose breakdown. This change of pH could not be prevented by increased buffering, as this in itself inhibited the enzymes concerned; thus increasing the phosphate concentrations from 0·03 to 0·3 M was sufficient to cause complete inhibition of gas production.

That the cessation of gas production was due to decrease of pH was proved by two procedures: (*a*) the addition of alkali from the side-arm of the vessel at the moment when the rate of gas production had reached a negligible figure; (*b*) a comparison of the duration of the linear production of hydrogen with different concentrations of bacteria.

(*a*) In experiments at 37° the evolution of H_2 from glucose usually ceased abruptly after 30–60 min., the pH value of the reaction mixture then being *c.* 5. When, just before this point was reached or immediately after, enough NaOH (usually 0·2 ml. 0·4N) was tipped in from the side-arm of the Warburg vessel to restore the pH to 7·0, H_2 production was immediately resumed at a rate approaching that observed initially. When the adjustment of the pH value was delayed, the recovery was progressively less complete, showing that the inactivation by acidity rapidly became irreversible (Fig. 1A and B).

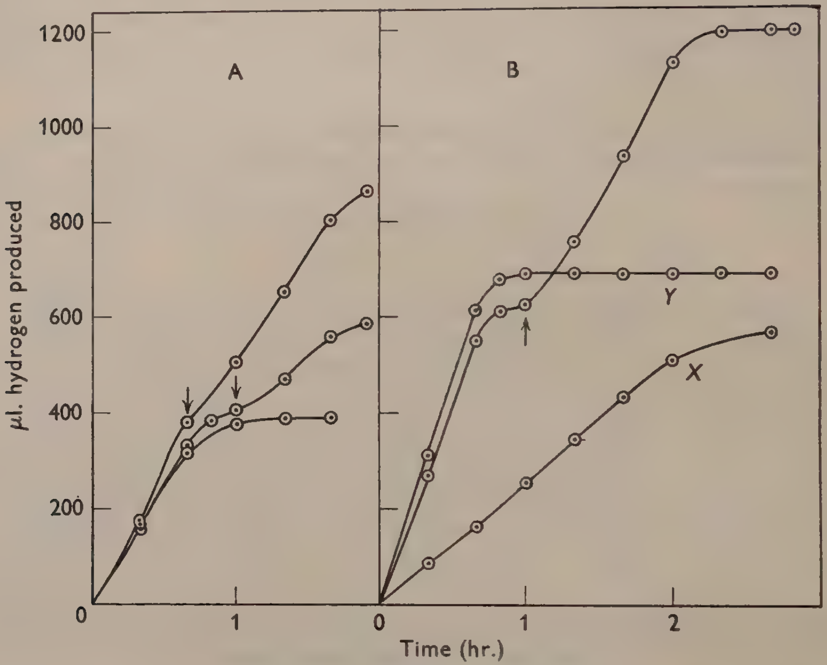

Fig. 1. The maintenance, or restoration, of hydrogen production from glucose by a suspension of washed bacteria of strain B6, brought about by addition of alkali. At the points marked by an arrow, 0·2 ml. 0·4N-NaOH was added to the Warburg vessel from the side-arm. Curve *X* (B) shows the course of hydrogen production by a quantity of bacteria one-half of that used to produce curve *Y*.

(*b*) With a more dilute bacterial suspension the acidification of the medium was slower; under these conditions the reaction was more prolonged, and sometimes continued linearly for several hours before it was stopped by the change of pH (Fig. 1B curves X and Y).

The attempt to determine the stability of glucose hydrogenlyase at the higher temperature was clearly frustrated by the effect of the accompanying pH change, so it was thought worthwhile to determine the stability of this enzyme in the absence of glucose, although in general enzymes are less stable to adverse conditions in absence of substrate. Suspensions of strains B6 and C59 in dilute buffer, having been grown in glucose broth at the normal temperature (for enzyme production), were held at the higher temperature for various periods of time, and then tested manometrically for their glucose hydrogenlyase content (Fig. 2). The results indicated different degrees of stability of the enzyme in the two strains. The enzyme was relatively stable for 2 hr. but was completely inactive after *c.* 4 hr. at the higher temperature. Two curious points noted in these experiments were: (i) in cases where the enzyme had been partially heat-inactivated, it showed a very rapid and quantitative recovery when incubated in glucose at the lower temperature; (ii) hydrogenase, far from being destroyed at the same time as the hydrogenlyase, showed an increase in apparent activity, sometimes as much as fivefold. These two observations were further investigated.

Recovery of glucose hydrogenlyase activity in suspensions in which this enzyme had been lost through heating

Suspensions grown at the normal temperature in glucose broth and not heated at the higher temperature, when tested for glucose hydrogenlyase at pH 7 showed an initially linear H_2 production, which then gradually accelerated by some 30 % as the pH value dropped to the optimum (pH 6·2). Similar suspensions heated at the higher temperature for *c.* 2 hr. showed a similar linear gas production; after 4 hr. heating they showed a decrease of *c.* 66 % in initial velocity, with a rapid recovery during the first 30 min. to a speed equal to that of the unheated control. After 6–8 hr. of heating the loss of activity was usually 90–100 %; the recovery then showed a lag of about 1 hr., and was not complete until 2 hr. from the beginning of the experiment. With longer heating the lag was longer, but the velocity ultimately attained was of the same order. These points are illustrated in Fig. 3. When formate was used as substrate instead of glucose, the disappearance of the enzyme took place at a similar rate, but no recovery was ever observed.

Further experiments were directed to determine whether this recovery of enzyme activity was associated with growth. For this purpose viable counts and total cell protein determinations were carried out on suspensions before and after the experiment. The viable count always showed a decrease and the total cell protein determination no change during the recovery period (Table 3). The absence of change in total bacterial protein precludes satisfactorily any increase in the total mass of bacteria present, but does not exclude the possibility that a few cell divisions had occurred. This could best have been tested by carrying out total cell counts, but this was rendered difficult and inaccurate by the clumping of cells which took place at the rather acid pH values reached in these experiments. The same reason might account for the apparent decrease in viable count, which was not expected.

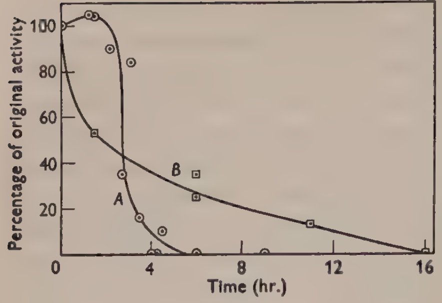

Fig. 2

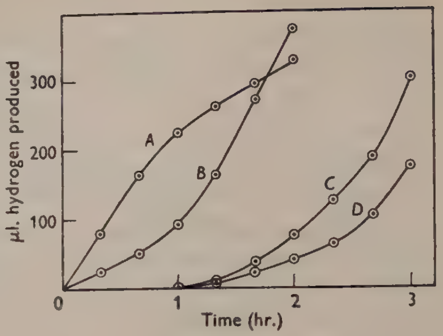

Fig. 3

Fig. 2. The rate of loss of glucose hydrogenlyase activity of suspensions of bacteria heated at 7° above the normal growth temperature. Curve *A*, strain C59 heated at 44°; curve *B*, strain B6 heated at 37°.

Fig. 3. The recovery of glucose hydrogenlyase activity in bacterial suspensions in which this activity had been destroyed by heating. Strain C59. Curve *A*, unheated suspension; curve *B*, suspension heated 3½ hr. at 44°; curve *C*, suspension heated 6 hr. at 44°; curve *D*, suspension heated 9 hr. at 44°.

Table 3. *Viable count and total protein content of heated and unheated suspensions of organisms* B6 *before and after the recovery of the glucose hydrogenlyase at* 30°

	Experiment 1		Experiment 2	
	Viable count (cells/ml.)	Protein content (mg./3 ml.)	Viable count (cells/ml.)	Protein content (mg./3 ml.)
Before heating 6 hr. at 37° (A)	25×10^9	1·18	17×10^9	1·75
After heating 6 hr. at 37° (B)	25×10^9	1·16	13×10^9	1·70
A after removal from Warburg vessel	20×10^9	1·17	6×10^9	1·77
B after removal from Warburg vessel	20×10^9	1·17	8×10^9	1·66

Increase in hydrogenase activity during heating

In suspensions of organism C59 or B6, grown in glucose broth at the normal temperature, the activities of formic dehydrogenase and of hydrogenase were roughly equal. When either organism suspended in dilute phosphate buffer at pH 7·0 was heated to 7° above its normal temperature, the formic dehydrogenase activity either remained constant or sometimes showed a very small increase. The hydrogenase activity, on the other hand, simultaneously with the complete disappearance of formic hydrogenlyase, showed a remarkable increase, amounting usually to 300% after 6 hr. of heating. This increased activity persisted after more prolonged heating, even up to 16 hr. (Fig. 4).

Affinity of formic dehydrogenase and formic hydrogenlyase

Much evidence has now been accumulated (Umbreit, 1951) which tends to indicate that formic hydrogenlyase is not an individual enzyme, but rather represents a linking of formic dehydrogenase and hydrogenase through a postulated and so far unidentified hydrogen carrier. In our experiments the simultaneous absence of hydrogenlyase and hydrogenase from suspensions grown at a temperature above the normal, points in the same direction. The disappearance of hydrogenlyase concurrently with a fivefold increase in hydrogenase does not fit so well, but could be explained as an effect of the higher temperature on the hydrogen-transporting coenzyme, although it would be unusual to find a coenzyme more heat-labile than the enzymes it linked together.

Evidence concerning this may be obtained from a consideration of the relationship between formate concentration and the rates of the two processes, viz. transfer of hydrogen from formate to carrier (formic dehydrogenase, using methylene blue in place of the intracellular carrier) and evolution of molecular hydrogen (formic hydrogenlyase). If in each process formic dehydrogenase were the enzyme concerned in the first attack on the formate, then this relationship would be expected to be the same for the two processes. The relationship can be expressed numerically as the concentration of substrate at which half the maximum velocity of reaction is reached (Michaelis constant; K_m); the reciprocal of this constant is a measure of the affinity of the enzyme for its substrate.

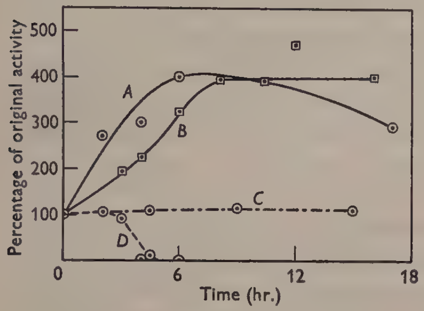

Fig. 4

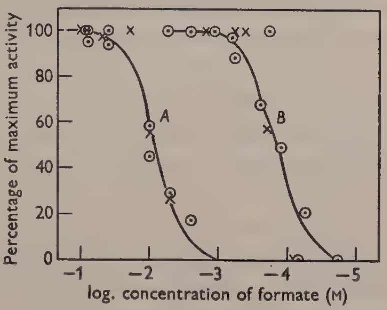

Fig. 5

Fig. 4. Increase in hydrogenase activity of suspensions of bacteria heated at 7° above normal temperature. Curve *A*, strain B6 heated at 37°; curve *B*, strain C59 heated at 44°. For comparison are included the effects of similar heating on formic dehydrogenase (curve *C*) and hydrogenlyase (curve *D*), both for strain B6.

Fig. 5. Comparison of the affinities of formic dehydrogenase and formic hydrogenlyase. Curve *A*, hydrogenlyase; curve *B*, dehydrogenase; ⊙, strain B6, 30°; ×, strain C59, 37°.

The affinities of formic dehydrogenase and formic hydrogenlyase were determined in *Escherichia coli* by Stephenson & Stickland (1931) and found to be far from equal. These values have now been redetermined for strains B6 and C59, and the 100-fold difference in affinity between the two enzymes confirmed (Fig. 5).

DISCUSSION

The original purpose of this study, namely to find why certain strains of bacteria fail to produce gas when they are grown at a temperature 7° higher than their normal growth temperature, has not been very far advanced. All that can be said is that the enzymes responsible for production of H_2 from glucose are fairly stable at the 'higher' temperatures of the organisms used here, and the failure to produce gas must be attributed to a failure of those mechanisms which produce the adaptive hydrogenlyase system. Bacteria which have no hydrogenlyase activity have no hydrogenase activity either; how far these two phenomena may be related will be further discussed below.

The history of the theories on the nature of formic hydrogenlyase has been recently reviewed by Umbreit (1951). That more than one hydrogenlyase exists has been confirmed by Koepsell & Johnson (1942), who demonstrated that formic acid was not an intermediate in the liberation of H_2 from pyruvic acid by preparations from *Clostridium butylicum.* Present opinion is, in the main, in favour of a duplex theory, namely that hydrogenase and formic dehydrogenase are linked by a hydrogen-transporting coenzyme to constitute 'formic hydrogenlyase' which has no existence as a separate entity. Several new arguments can be offered which tend to weaken this theory.

(*a*) Such a hydrogen-transporting coenzyme would have to have an oxidation-reduction potential in the neighbourhood of that of the hydrogen electrode; it seems unlikely that any haematin derivative or other iron-containing coenzyme would have such a potential. The evidence derived from the effects of Fe-deficient media (Waring & Werkman, 1944) seems therefore to be irrelevant.

(*b*) In many circumstances in which *Escherichia coli* is deficient in formic hydrogenlyase it is also deficient in hydrogenase. In our experiments this is confirmed for bacteria grown at the higher temperatures. However, there are two important exceptions: (i) in bacteria grown aerobically at normal temperature, i.e. unadapted; here it must be assumed that, as hydrogenase and formic dehydrogenase are both present in normal amounts, the postulated coenzyme is missing; (ii) in our experiments in bacteria grown at normal temperatures in glucose so as to contain formic hydrogenlyase, and then heated at the higher temperature until the hydrogenlyase activity has been destroyed. In such preparations formic dehydrogenase is quite stable and hydrogenase is actually increased in activity; therefore it must be assumed, on the duplex theory, that the postulated hydrogen carrier has been destroyed. We should have then the curious case of a coenzyme which was more heat-labile than the enzymes it linked.

(*c*) The affinity of formic dehydrogenase for its substrate is very high (K_m is of the order of 10^{-4} M). If in each case the first action were between formic dehydrogenase and its substrate, the affinities should be the same and the subsequent fate of the hydrogen should not affect it. In fact, the affinity of formic hydrogenlyase is much less; $K_m = 10^{-2}$ M. Lichstein & Boyd (1953) produced evidence indicating that under certain conditions *Aerobacter aerogenes* can show formic hydrogenlyase activity while it contains no hydrogenase. This they took as proof of the separate existence of a formic hydrogenlyase; but the evidence is not entirely convincing as the enzyme tests, in the case of formic hydrogenlyase, involved incubation of the organisms with hydrolysed yeast extract and formate which were absent from the test for hydrogenase. It is not impossible that in the suspension incubated for 30 min. with formate and hydrolysed yeast extract, hydrogenase, too, might have been regenerated.

The nature of the recovery of the power to produce hydrogen from glucose, by bacteria which had lost this power through incubation at the higher temperature, deserves some comment. These bacteria resemble unadapted bacteria in their complete lack of hydrogenlyase. Unadapted bacteria, however, cannot acquire the power of hydrogen production except by incubation with glucose (or formate) + an amino acid mixture (Stephenson & Stickland, 1932; Pinsky & Stokes, 1952). Pinsky & Stokes suggested that the process of adaptation involves synthesis of enzyme protein from the amino acids supplied (or, if the duplex theory of hydrogenlyase be accepted, synthesis of the protein portion of the hydrogen-transporting carrier; cf. Yudkin, 1938; Mandelstam, 1952). Bacteria rendered devoid of hydrogenlyase through heating, on the other hand, show rapid recovery of enzyme when incubated with glucose alone (though not with formate alone). Their deficiency is therefore clearly not of the same nature as the deficiency in unadapted bacteria. The possibility that sufficient amino acids were liberated into the suspension during heating to allow readaptation to take place when the temperature was restored to normal was excluded by experiments in which the heated suspension was thoroughly washed before the recovery experiment; the recovery was only very slightly delayed by this treatment.

The change undergone by the hydrogenlyase system when inactivated by heating at 7° above the normal temperature is less thorough-going, not involving destruction of protein, and is reversible at the normal temperature in the presence of glucose (but not formate). The kind of change that can be imagined is the splitting of a prosthetic group from a protein, or the oxidation or dephosphorylation of a prosthetic group to an inactive form. Glucose might reverse these changes by providing energy, or by providing strongly reducing conditions; the failure of formate to reverse the changes suggests that glucose acts by providing energy.

One of us (J.G.) is indebted to the Medical Research Council for a grant for expenses in aid of this work.

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Note. While this paper was in the press, results by Gest and his collaborators have appeared (Gest 1954) which offer further, but still inconclusive evidence for the duplex nature of formic hydrogenlyase.

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Genetic Investigation of the Inhibition by DL-Norleucine of the K-12 Strain of *Escherichia coli*

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SUMMARY: The genetic relationship to other markers of the locus for inhibition of K-12 strain of *Escherichia coli* by norleucine has been investigated. The results showed little or no linkage with the loci for methionine, leucine, threonine or thiamine synthesis, but a close association was found with the Gal_5^- and Arab^- loci of strain W-677.

It is probable that micro-organisms, with their presumably limited numbers of genes, will provide some of the most fruitful material available for the study of inter-action of neighbouring genes. One important aspect of this problem is whether or not the fact that two genes are adjacent on the chromosome has any biochemical significance. The mapping of the positions of various genetic loci of *Escherichia coli* strain K-12 is proceeding steadily, but it has not yet exceeded a purely descriptive level. One possible instance of closely linked loci having physiologically related biochemical effects has recently been reported (Manten & Rowley, 1953); the locus for resistance to inhibition by valine in K-12 was shown to have a very high degree of linkage with that for the independence of an antagonizing amino acid, leucine.

The K-12 strain is also inhibited on minimal medium by low concentrations of norleucine, and this inhibition can be prevented by the simultaneous presence of methionine (Rowley, 1953). By marking K-12 with resistance to norleucine the genetic linkage of the locus for this character to other markers may be studied. In particular, the possibility of its close linkage with methionine independence, a tempting speculation by analogy with the linkage of valine resistance with leucine, can be investigated.

MATERIALS AND METHODS

The strains of *Escherichia coli* used were:

(*a*) K-12/F+ (wild-type strain). This grows well in minimal medium, unsupplemented with amino acids or vitamins. Since this strain mates with F− strains, it has been presumed to be F+ (Hayes, 1953; Cavalli, Lederberg & Lederberg, 1953). It is inhibited by valine (0·1 μg./ml.) which is reversed by either leucine (0·05 μg./ml.) or isoleucine (0·01 μg./ml.), and also by norleucine (1 μg./ml.), which is reversed by methionine (0·2 μg./ml.). A stable mutant of this strain ($\mathrm{K\text{-}12/Nl^R}$) resistant to norleucine at concentrations up to 1 mg./ml., but still sensitive to valine, was selected by plating large inocula of the wild type K-12 strain on plates of minimal agar containing 1 mg./ml.

norleucine. Both strains are capable of fermenting maltose, lactose, D-galactose, D-xylose, D-mannitol and L-arabinose (Mal+, Lac+, Gal+, Xyl+, Mtl+, Arab+), shown by the production of characteristic blue-black colonies with a metallic lustre on eosin+methylene blue (EMB) agar containing any of these sugars.

(*b*) 58–161. A mutant of K-12 requiring methionine (20 μg./ml.), (M−). F+ and F− forms of this strain were used and also streptomycin-resistant mutants of both forms (58–161/St^R/F− and 58–161/St^R/F+). All these strains ferment the same sugars as the parent K-12 strain.

(*c*) W-677. A mutant of K-12 requiring threonine (20 μg./ml.), leucine (20 μg./ml.) and thiamine (20 μg./ml.), (T$^-$, L$^-$, B$_1^-$). W-677/F+, W-677/F−, W-677/St^R/F+ and W-677/St^R/F− were used, none of which fermented the carbohydrates already mentioned, as evidenced by the production of pink colonies on EMB agar supplemented with these carbohydrates. The W-677 strains can be designated Mal$^-$, Lac$^-$, Gal$^-$, Xyl$^-$, Mtl$^-$, Arab$^-$.

(*d*) J5–10/F+. A K-12 mutant requiring proline (20 μg./ml.) and histidine (20 μg./ml.) (P$^-$, H$^-$). This strain mates with F− strains and is thus implied to be F+. By plating heavy inocula on supplemented nutrient agar plates containing streptomycin (250 μg./ml.) or minimal agar plates containing norleucine (1 mg./ml.), strains resistant to streptomycin (J5–10/St^R) and norleucine (J5-10/St^R/Nl^R, J5-10/Nl^R) were obtained. The J5–10 strains are Mal+, Lac+, Gal+, Xyl+, Mtl+, Arab+.

Media

The *minimal medium* (MM) of Davis & Mingioli (1950) was used throughout, supplemented with glucose or other carbohydrates. Minimal media supplemented with growth factors, e.g. MM+B$_1$, MM+T+L, was used in certain matings.

EMB agar used in sugar fermentation tests was made up as detailed by Lederberg (1950), incorporating one of the following carbohydrates: maltose, lactose, D-galactose, D-xylose, D-mannitol or L-arabinose (supplied by Thomas Kerfoot and Co., Vale of Bardsley, Lancashire).

Nutrient agar and *nutrient broth* were those routinely employed in this Institute and were prepared from a tryptic digest of beef.

Technique of recombination tests

Auxotrophic technique. The two auxotrophic parent strains were grown overnight in nutrient broth at 37°, and, after diluting with 4 vol. of fresh broth, reincubated for 4 hr. Equal volumes of both parent cultures were then mixed, centrifuged, and the bacterial deposit resuspended in minimal medium. This procedure was repeated twice and the volume finally adjusted to 1/10 that of the original; 0·1 ml. samples were then spread over the surface of minimal or supplemented minimal agar plates in quintuplicate. Controls in duplicate of each parent were washed, concentrated, and plated separately. Colonies were isolated from the plates spread with the mixed culture after 40–48 hr. incubation at 37°, the 'parental' control plates being invariably devoid of colonies.

Streptomycin prototroph technique (SRP). Overnight cultures of the parent strains, one of which was auxotrophic and streptomycin-resistant and the other prototrophic and sensitive, were diluted in fresh broth and equal volumes of the parental cultures were mixed and incubated at 37° for 4 hr. After washing and concentrating as above, samples were plated on minimal medium containing streptomycin (250 μg./ml.), (MM + St).

Purification of colonies

The surface of recombinant colonies was touched with a sterile loop and an inoculum emulsified in 0·5 ml. sterile saline to give a slight visible turbidity. A loopful of the suspension was then applied to the surface of an agar plate containing the same supplements as that from which the colony was selected. By positioning over a template, fifty colonies could be accommodated on a single agar plate which, after overnight incubation at 37°, was used as a source for further tests. In this way, contamination from the 'background' of the parental strains was minimized.

Sugar fermentation tests. A faintly turbid dilution in saline of each purified recombinant colony was then applied to the surfaces of EMB or MM plates containing the appropriate carbohydrate to be tested, whilst positioned over a template, so that each colony was applied on the same position on each plate.

Streptomycin and norleucine sensitivity. A loopful of the same dilution of purified recombinant colonies was streaked on freshly prepared plates of minimal agar up to ditches, containing either streptomycin (250 μg./ml.) or norleucine (500 μg./ml.). After incubation for 24 hr., sensitive progeny were inhibited approximately 1 cm. from the ditch, whereas resistant progeny showed growth along the whole length of the streak.

RESULTS

Inhibition of parent strains

K-12 and W-677 are inhibited by norleucine when tested on appropriately supplemented norleucine ditch plates (500 μg./ml.). 58–161 shows no such inhibition. This may be due, however, to the antagonism of norleucine inhibition by the methionine which is necessary to allow growth of this auxotroph. K-12 is also uninhibited by norleucine on this methionine-supplemented medium.

The results in Table 1 show that when the F− form of 58–161 is mated with a norleucine-resistant strain a proportion of the progeny are sensitive to norleucine, indicating that 58–161 carries the gene determining sensitivity to norleucine, although its action is undetectable on the methionine-containing media required for growth of this strain.

Investigation of linkage to selected markers

From the work of Hayes (1952), it is evident that in recombination there is normally a one-way transfer of genetic material. The strain which acts as a gene donor has been designated as F+ and the strain acting as a gene acceptor as F− (Lederberg, Cavalli & Lederberg, 1952). Matings can occur only when at least one of the strains is F+. The F+ strain, when mated with an F− strain, can be envisaged as contributing that part of its chromosome (or chromosomes) required by the F− parent to satisfy the requirements of the selecting medium.

When 58–161/St^R/F− is mated with K-12/Nl^R/F+ using the SRP technique only 1·5 % of the progeny are resistant to norleucine (Table 1, line 6). According to the above hypothesis, the F+ parent (K-12/Nl^R/F+) contributes to the recombinants selected for on MM+St that part of the chromosome which carries the factor for methionine synthesis (M+). Since the factor for norleucine resistance also carried by the F+ strain is transferred to such a small extent to these recombinants, we can deduce that there is little linkage between these two factors, or that if the Nl locus is linked to the M locus, this linkage is concealed by its closer linkage to a selected marker of the F− parent, in this case the locus for streptomycin resistance. The lack of linkage between Nl^R and M+ is corroborated by mating a Nl-resistant mutant of J5–10/F+ with 58–161/F− when of 100 progeny examined none was found to be Nl-resistant.

Table 1. *Summary of matings and sensitivity of progeny to norleucine inhibition*

Mating	Medium of selection	Loci selected from F+ parent	No. of colonies tested	Colonies resistant to Nl (%)
1. 58–161/F+ × W-677/F−	MM	T, L, B_1	192	0
2. J5–10/F+ × 58–161/F−	MM	M	142	0
3. 58–161/F+ × J5–10/F+	MM	M or P, H	135	0
4. J5–10/Nl^R/F+ × 58–161/F−	MM	M	100	0
5. J5–10/Nl^R/St^R/F+ × W-677/F−	MM	T, L, B_1	305	7
6. K-12/Nl^R/F+ × 58–161/St^R/F−	MM+St	M	276	1·5
7. K-12/Nl^R/F+ × W-677/St^R/F−	MM+St	T, L, B_1	227	0
	MM+St+B_1	T, L	183	0
	MM+St+T+L	B_1	83	0
8. K-12/Nl^R/F+ × 58–161/St^R/F+	MM+St	St or M	206	13
9. K-12/Nl^R/F+ × W-677/St^R/F+	MM+St	St or T, L, B_1	56	25

Similarly, the absence of significant linkage between the locus of norleucine inhibition and those for threonine, leucine or thiamine synthesis is shown by the absence of Nl^R recombinants in the mating of K-12/Nl^R/F+ and W-677/St^R/F− when the three loci for T+, L+ and B_1+ are selected for by mating on MM+St. This applies equally when these factors are separated by mating on MM+St+B_1 (selecting for T+ and L+ only). The cross of J5–10/Nl^R/St^R/F+ × W-677/F− on MM yields only 7 % Nl^R recombinants which confirms the lack of any significant linkage of Nl with either T, L or B_1.

In these matings it is evident that the recombinants possess almost exclusively the norleucine genotype of the F− parent and no transfer of the norleucine locus has taken place. Matings were planned, therefore, in which the norleucine locus was selected for by incorporating the amino acid in the selecting medium. The progeny from this type of mating could then be examined for other unselected markers in order to compare their frequency with those from similar matings in which there was no selection for norleucine. Table 2 shows the analysis on EMB agar of the sugar fermentation markers of the progeny from the crossing of K-12/Nl^R/F+ and W-677/St^R/F− when seeded on to MM+St containing Nl (500 μg./ml.), which selects those recombinants in which a transfer from the F+ to the F− parent of the locus for norleucine resistance (Nl^R) has occurred as well as of the T+, L+ and B_1^+ loci. By further supplementing the recombination plates with growth factors, recombinants could be isolated in which the norleucine locus only is selected. (The distribution of sugar fermentations of progeny from the same parents without selection of norleucine resistance is provided as comparison.)

Table 2. *Effect of selection for locus for Nl on sugar fermentations of recombinants from the mating* K12/Nl^R/F+ × W-677/St^R/F−

Selecting medium	No. of colonies tested	Percentage recombinants having the phenotype						
		Nl^R	Mal$^+$	Lac$^+$	Gal$^+$	Xyl$^+$	Mtl$^+$	Arab$^+$
1. MM+St+Nl	50	100	14	70	86	14	18	80
2. MM+St+Nl+B_1	94	100	5	83	95	7	15	85
3. MM+St+Nl+T+L	143	100	15	50	98	25	28	82
4. MM+St+Nl+T+L+B_1	184	100	15	38	93	27	26	73
5. MM+St	226	0	4	26	1	13	12	0
6. MM+St+B_1	183	0	1	35	0	3	2	3
7. MM+St+T+L	83	0	1	31	0	2	4	0

K12/Nl^R is Nl^{R}St^{S}T$^+$L$^+$$B_1^+Mal^+Lac^+Gal^+Xyl^+Mtl^+$Arab$^+$.
W-677/St^R/F− is Nl^{S}St^{R}T$^-$L$^-$$B_1^-Mal^-Lac^-Gal^-Xyl^-Mtl^-$Arab$^-$.

The results show that selection for the locus for norleucine resistance only, as is effected when the mating is plated on minimal medium supplemented with St, Nl, T, L and B_1, involves an accompanying transfer of many of the sugar markers. From this it can be seen that Nl^R is linked with Gal$^+$ and to a lesser extent with Arab$^+$.

Further evidence supporting the linkage of the locus for Nl inhibition with that for galactose fermentation is provided by the mating of K-12/Nl^R/F+ and W-677/St^R/F+ indicated in Table 1, line 9. In this mating there is no selection for Nl. Nevertheless, from fifty-six colonies tested fourteen were Nl resistant and each of these, and these only, was also Gal+.

Further investigation of Gal$^+$ and Nl loci

On selecting for the loci of T+, L+ and B_1^+ in the cross K-12/Nl^R/F+ × W-677/S^R/F−, no transfer of Nl^R and consequently no linkage with these factors was found. Similarly, from the inability to transfer Nl^R to any significant extent in the cross K-12/Nl^R/F+ × 58–161/St^R/F−, the inference is made of lack of linkage between Nl^R and M$^+$. From the work of Watson & Hayes (1953), it appears that there are at least three linkage groups in the K-12 chromosome identified with the markers TL/Lac, M/B_1 and St/Mal. With the inability to localize Nl with either of the first two groups, and the fact that the Gal-locus of W-677 is also unlocalized, the possibility of the linkage of Nl and Gal factors to those of the third group, St-Mal, must be considered. For this it is necessary to call over one of the markers in the St-Mal group from the F+ to the F− parent and score the progeny for the other markers.

In this investigation, 58–161/St^R/F+ and J5–10/Nl^R/St^R/F+ were each crossed with W-677/F−. According to Hayes (1953), although St^SF+ × St^RF− crosses are productive on MM+St, the fertility of St^RF+ × St^SF− crosses is negligible. We found that recombinants do arise from the latter type of mating (and their number can be increased by supplementing with the F− growth factors), but the numbers of recombinants are only 2–3 % of the streptomycin-resistant colonies arising on minimal agar. The progeny from the cross J5–10/St^R/Nl^R/F+ × W-677/F− selected on minimal medium, and so transferring the factors T^+, L^+ and B_1^+, were scored for St^R and Nl^R. Transfers of 29 % St^R, 24 % Mal^+, 7 % Nl^R and 9 % Gal^+ were found. Among these St^R progeny, 17 % were Nl^R and 21 % were Gal^+ and among the St^S progeny, 4 % were Nl^R and 3 % Gal^+. This increase in proportion of Gal^+ among St^R progeny is substantiated in the mating 58–161/St^R/F+ × W-677/F−, where although the transfer of T^+, L^+ and B_1^+ brings over only 10 % St^R and 3 % Gal^+, among these St^R progeny there are 27 % which are also Gal^+. This indicates that there is a certain amount of linkage of the Nl and Gal loci with St.

DISCUSSION

The present work demonstrates the lack of linkage between the loci for resistance to norleucine inhibition (Nl^R) and the ability to synthesize methionine (M+). The previously reported linkage between resistance to valine and independence of leucine may therefore be coincidental. However, since leucine has been shown to antagonize valine only, whereas methionine is reported to antagonize inhibition by norvaline, lysine and cysteine (Rowley, 1953), the lack of linkage between Nl^R and M^+ may be due to the non-specificity of methionine antagonism. The evidence that methionine synthesis is interrelated with that of many other amino acids and that its role in bacterial metabolism is fundamental and complex lends support to this possibility.

The linkage of the sugar markers to Nl is seen most clearly by comparison of results obtained by selecting for this locus with those when selection is not made (Table 2). This indicates an association of Nl with all the sugar markers, particularly the loci for Gal and Arab.

It will be noted from the results shown in Table 2 that the pattern of sugar markers differs from that previously published by Cavalli *et al.* (1953) for similar matings. These authors investigated the distribution of sugar markers from the mating of a $T^+L^+B^+$/St^S/F+ strain with a $T^-L^-B_1^-$/St^R/F− strain (W-945). The percentage transfer of sugar markers (scored on EMB) was found to be Arab 99·5 %, Lac 58 %, Gal 32 %, Xyl 25 % and Mal 10 %, our results from the same type of mating (Table 2, line 5) being Arab 0 %, Lac 26 %, Gal 1 %, Xyl 15 % and Mal 4 %. The strain W-945 carries the markers Mal_1^-, Lac_1^-, Gal^-, Xyl^-, Mtl^-, $Arab^-$ and was derived from the same parent W-1 (T^-, L^-, B_1^-, Lac_1^-, Mal_1^-) as W-677. Thus the loci Lac_1^-, Mal_1^-, are allelic in the two strains, but since the markers Gal^-, Xyl^-, Mtl^- and $Arab^-$ were built in separately these may or may not be allelic (E. M. Lederberg, personal communication). This possibility of non-allelism in these sugar markers led us to investigate the inability of strain W-677 to ferment these sugars. The locus Gal_5^- carried by W-677 allows slow growth of this strain on galactose MM (Wollman, 1953). On testing W-677 on MM incorporating either lactose, maltose, xylose, mannitol or arabinose as the sole carbohydrate source, no growth was observed except on arabinose. Strain W-945, however, was found to be unable to ferment any of the six sugars. This difference in phenotypic expression implies that the Gal^- and $Arab^-$ loci may be non-allelic in these two strains. This has been confirmed by crossing $W\text{-}945/St^R/F+$ with $W\text{-}677/Az^R/V_1^R/F-$ on MM supplemented with T, L, B_1, St, Az and phage T_1, when 5–8 % of the recombinants were Gal^+ and 20–25 % were $Arab^+$, (L. L. Cavalli-Sforza—personal communication). The locus for fermentation of galactose carried by strain W-945 has since been characterized as Gal_2^- (E. M. Lederberg, personal communication). Hence these differences between our results (Table 2) and those of Cavalli *et al.* (1953) are presumably due in the case of Gal and Arab to the non-allelism of these loci in the two strains. It is the Gal_5^- and $Arab^-$ loci of strain W-677 that are linked with Nl. Of the three linkage groups suggested by Watson & Hayes (1953), the only one to which Nl shows any significant linkage is that carrying the St-Mal loci. The linkage with this group is however small, and the possibility remains that the Nl locus may lie in another linkage group altogether.

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Isolation of Nutritional Mutants of *Neurospora crassa* by Filtration Enrichment

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SUMMARY: Nutritional mutants of *Neurospora crassa* may be isolated by incubation of microconidia in a liquid medium deficient in specific growth factors followed by filtration and then growth on supplemented media of the conidia remaining in the filtrate.

A method for the isolation of nutritional mutants of *Neurospora crassa* by filtration and subsequent selective enrichment of the medium has been developed as an adaptation of the one devised by Fries (1947) for *Ophiostoma multiannulatum.* It involves the use of a strain which produces microconidia abundantly, and preferably a strain that will grow as a colony or respond well by growing as a colonial paramorph on a suitable medium (Tatum, Barratt & Cutter, 1949). The significance of the use of microconidial strains is that only in a microconidium will a mutant nucleus have a chance of growing ultimately into a colony with the mutant character. A macroconidium containing a mutant nucleus would germinate and grow on minimal medium by virtue of the presence of one or more non-mutant nuclei, and so would be discarded in the course of the operations designed to eliminate as many as possible of the conidia which contain non-mutant nuclei. Stocks of *N. crassa* such as Y 8743, which is colonial and peach-microconidial (Barratt & Garnjobst, 1949), or the peach fluffy double mutant, which is not colonial, are suitable. They should be grown on a medium which induces the production of a large number of conidia. Some strains produce microconidia almost exclusively if the inoculum is buried under a layer of agar. The mycelium grows through this and produces a dense crop of microconidia on the surface.

The microconidia are harvested by washing them off the surface of the culture with sterile distilled water. The suspension is then filtered by means of a sterile cotton-wool filter to remove fragments of mycelium, yielding a dense opalescent suspension of microconidia. The filter is made by blowing a hole in the bottom of a Pyrex or other hard glass test-tube, and fusing to this hole a short length of Pyrex tube of about 7 mm. diameter to form a drainage stem. A small piece of absorbent cotton-wool is inserted into the base of the filter and just within the drainage stem. The open end of the filter is plugged with non-absorbent cotton-wool and a wrapping of this is also made round the stem and inserted into an intact test-tube. The whole assembly is then autoclaved.

The filtered suspension is irradiated with ultraviolet light by either of two methods: (1) the suspension is transferred to a sterile Petri dish and rocked with the cover off under a suitable ultraviolet lamp; (2) the suspension is transferred to a sterile quartz tube which is then sealed with a sterile cork so that not less than one-eighth of the volume is occupied by air; the tube is clamped in a suitable arrangement so that it is held horizontally and may be rotated continuously about this axis under a suitable ultraviolet lamp. The exposure time must be found for a given apparatus by trial.

The lamp used was a low pressure mercury vapour burner T/M5/369, obtained from the Thermal Syndicate Ltd., Vitreosil Works, Wallsend-on-Tyne. Two minute exposure at a distance of 15 cm. was used for *Neurospora crassa* microconidia, 30 sec. for haploid yeast and 8 min. for *Chromocrea spinulosa* ascospores.

After irradiation, samples of the conidial suspension are transferred to 100 ml. Erlenmeyer flasks containing 20 ml. liquid minimal medium. Westergaard & Mitchell's formula (1947) is a suitable one, but may have to be modified if the fungus grows too vigorously. The substitution of sorbose for some of the glucose or sucrose helps to decrease the growth rate, but the optimal proportions must be found empirically for a given stock. Commonly, about 0·8 % sorbose, together with 1 % sucrose, gives a convenient diminution of growth rate. However, a lesser proportion of sucrose may be needed in the plating medium referred to later.

The flasks are placed in a shaking machine and agitated continuously while being incubated at 25° or at a higher temperature when temperature-sensitive mutants are sought. Agitation should be fairly gentle, the flasks being moved in each direction about once a second. Too violent agitation may throw liquid on to the plug where the fungus may grow and conidiate and so contaminate the suspension with further unwanted conidia. After not more than 24 hr. the contents of each flask are filtered through a sterile cotton-wool filter into a fresh sterile Erlenmeyer flask.

During the period of incubation many of those conidia which are capable of growth in the minimal medium form small spherical colonies. These colonies do not pass through the cotton-wool filter but the great majority of the ungerminated conidia do. The flasks containing the filtrate are returned to the shaker and incubated for further periods, each followed by filtration, the intervals between successive filtrations being adjusted according to the rapidity and amount of growth, until no more visible growth occurs in the flasks. This normally requires 3 or even 4 days of incubation, since after irradiation a number of conidia, ultimately capable of germinating in minimal medium, are delayed, sometimes for more than 2 days.

When little or no further growth appears, samples of the remaining suspension, after a final filtration, are plated in Petri dishes containing agar medium of composition similar to that employed in the flasks, but supplemented with the factor or factors for which nutritionally deficient mutants are sought. When colonies have grown after incubation, transfers are made to supplemented slopes and the isolates are tested individually. The usual experience is that a number of the isolates are non-mutants, delayed in germination, but an appreciable proportion of the isolates are of the kind desired.

Outcrosses and re-isolation are necessary to rid the stocks of undesirable characters, such as colonial growth and microconidial habit, which were essential in the stock to provide uninucleate conidia.

Table 1 summarizes some of the experiences in the use of the filtration enrichment method with *Neurospora crassa* stock Y 8743. The first line of the Table illustrates the very low yield of mutants obtained when no selective enrichment is practised, but instead the irradiated conidia are plated out in suitable dilution and every colony tested individually.

Table 1. *Yields of nutritional mutants from total isolation and filtration enrichment*

Method	Supplement used	No. of expts.	Total isolated	Mutants obtained
Total isolation	Malt extract	10	1691	9
Filtration enrichment	Malt extract	4	273	39
	Histidine	1	43	3
	Ammonium salt	2	41	4
	Casein hydrolysate	1	31	4
	Adenine	1	44	3
	Proline	1	105	2
	Methionine	1	47	3

The filtration enrichment method works most satisfactorily for mutants which require a considerable amount of growth factor to achieve appreciable growth. For those mutants which require a relatively very small amount of supplement, such as those which require a vitamin, centrifugation and washing to remove growth factors liberated during growth or by conidia killed during the irradiation may be essential to success. The method is adaptable to a number of particular problems by suitable modification of the medium used during the shaking process.

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A Study of the Diffusible Lipase produced by Staphylococci and of its Immunological Activity

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SUMMARY: A systematic investigation of eighteen genera showed that staphylococci were the only potentially pathogenic group which produced a diffusible lipase detectable on solid culture medium. This lipase was obtained in filtrates of cultures grown by a well-established method for preparing staphylococcal toxins. When titrated, filtrates of very active strains showed lipolytic activity in dilutions of 1/4096, but the majority of strains had a titre of 1/256.

Whole cultures and culture filtrates of albus strains and formolized filtrates of aureus strains were found to stimulate the production of anti-lipolytic antibody in rabbit serum. The titre of the most active antiserum was 1/40, and that of the majority 1/10. Each antiserum prepared showed maximal neutralizing activity against the homologous filtrate, but all antisera studied neutralized heterologous filtrates to some extent. No evidence of naturally occurring anti-lipolytic antibody was found in any of the rabbit sera used.

A considerable amount of work has been done on the lipolytic activity of micro-organisms concerned in food-spoilage, but relatively little on that of potential pathogens. Among the earliest workers were Escherisch (1886) and Nencki (1886), from whose observations numerous subsequent workers developed techniques to demonstrate the ability of micro-organisms to hydrolyse a wide range of fatty substrates; much of this work was reviewed by Jensen & Grettie (1937). The majority of organisms produced either the exo-lipase or the endo-lipase but some strains produced both types. The type of enzyme studied depended upon the technique used, the activity of endo-enzymes being demonstrated when physico-chemical treatment of the bacterial cells was employed, and that of exo-enzymes by culture methods with living cells. In the present work investigations were limited to the exo-lipases. A systematic survey covering over 1000 strains of eighteen genera showed that, among potentially pathogenic groups, only the staphylococci produced a diffusible or exo-lipase.

MATERIALS AND METHODS

Solid media were found to be more sensitive than fluid ones for demonstrating lipolytic activity of growing bacteria.

Fat emulsion. Horse fat was used because, in fatty-acid composition, it resembled human fat more closely than any other mammalian fat available (Hilditch, 1947). The emulsion was prepared from the following: horse fat 4·0 ml.; 6·0% (w/v) aqueous agar 3·0 ml.; distilled water 93·0 ml. The melted fat and aqueous agar were mixed and boiling water added gradually, the mixture being passed repeatedly through a hand-driven domestic mechanical emulsifier ('Cream Maker, Empire Series', British Emulsifiers Ltd., Greek Street, London, W.1).

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Nutrient fat agar (N.F.A.) was prepared by adding an emulsion of horse fat to beef infusion agar pH 7·2–7·4, to give a final concentration of fat 0·3 % (w/v). 7·5 ml. emulsion was added to 92·5 ml. melted agar. Twenty-five ml. of medium were poured into Petri dishes (7·0 cm. diam.) which were dried for 1 hr. before use. On this medium lipolytic activity was shown by a zone of clearing round the colony (Pl. 1, fig. 1).

Nutrient fat Nile Blue sulphate agar (N.B.S.A.). This medium differed from N.F.A. above only in containing 2·0 ml. aqueous 0·1 % (w/v) Nile Blue sulphate to every 100 ml. N.F.A. Nile Blue sulphate is an oxazine dye and is pink in the presence of neutral fat and blue with free fatty-acids (Kaufmann & Lehmann, 1926; Rettie, 1931; Knaysi, 1941; Govan, 1944). It was first used in media for observing lipolysis by Turner (1929). Strains which showed positive results on this medium produced a blue-coloured colony surrounded by a clear blue zone. Strongly positive strains produced a bright blue colour and a wide surrounding zone, while less active strains produced a paler colour and a narrower zone. This medium was found to be better than N.F.A. for studying culture filtrates.

Strains of staphylococci. Eighty-eight strains were studied, forty-eight of *Staphylococcus aureus* of which forty-one (85·4 %) were lipolytic, thirty-eight of *S. albus* of which twenty-nine (76·3 %) were lipolytic, and two of *S. citreus* of which one was lipolytic. Twenty-five of the *S. aureus* strains had been associated with outbreaks of food-poisoning and were obtained by the courtesy of Dr R. E. O. Williams (London) and Dr J. Marks (Cardiff). All other strains were isolated from pathological materials.

Control strains of bacteria. Several strains of *Escherichia coli* were used as negative controls during the isolation of lipolytic staphylococci. Non-lipolytic staphylococci were used when investigating staphylococcal lipase in culture filtrates.

Preparation of culture filtrates. The method recommended by Mackie & McCartney (1948) for the preparation of staphylococcal toxin was used. Semi-solid agar (agar 0·8 %, w/v), 25 ml. in 250 ml. conical flasks, was inoculated with 5·0 ml. of 24 hr. broth culture and incubated at 37° in an atmosphere of 25 % carbon dioxide for 3 days. Buffered peptone broth containing 3 % (w/v) peptone was added in the proportion of 5·0 ml. broth to 25 ml. semi-solid agar, and the flasks reincubated as before for a further 3 days. Cultures were harvested by filtering first through paper then through Chamberland filter candles.

Lipolytic activity of culture filtrates. This was tested by using N.B.S.A. and porcelain cylinders, according to the method of Heatley (1944). Five cylinders were fixed to each plate, the central position being reserved for the control fluid which had been heated at 85° for 45 min. During incubation at 37° the filtrate diffused from the cylinders and produced blue zones after 18 hr. of incubation. No zones were formed by the heated control fluids. When N.F.A. medium was used instead of N.B.S.A. corresponding colourless zones were formed, but were much less distinct. As incubation proceeded, up to 96 hr., the zones enlarged and concentric rings formed. The zones produced by all active filtrates showed a comparable pattern in their concentric rings. The central ring, or area immediately round the cylinder, was deep blue, relatively opaque and sometimes showed precipitation resembling the deposition of fatty-acid crystals. The ring immediately surrounding this area was clear and transparent, usually violet in colour but sometimes colourless. In some cases this ring divided into two, of which one was blue in colour. The third ring was always the narrowest, and was clear and colourless or had a pale pink tint. Sometimes another poorly defined colourless ring developed outside the narrow one (Pl. 1, fig. 2).

Titration of culture filtrates. Filtrates and dilutions up to 1/4096 were dropped from calibrated dropping pipettes (50 drops/ml.) over the surface of N.B.S.A. plates resting on a ruled template. When the drops were absorbed the plates were incubated at 37° for 48 hr. Zones of colour were formed and differentiated into concentric rings similar to those observed when porcelain cylinders were used. The titre of a filtrate was arbitrarily selected as that dilution which just failed to show differentiation into concentric rings after the plate had been incubated for 48 hr. This end-point was nearly always some 4–8 times the lowest dilution which showed demonstrable lipolysis. In the lower dilutions there was little variation in the size of the zones, but as the dilution approached the end-point, the size and intensity tended to decrease. The average of triplicate titrations was taken as the titre.

Properties of staphylococcal lipase. The optimal period for production of lipolytic culture filtrates was found by incubating the cultures for periods varying from 6 to 20 days. The optimal medium was found by growing strains in five batches of beef infusion broth, each of which gave a different final pH value after the growth of the same organism. The effect of the volume of medium on the production of lipase was studied by comparing the activity of filtrates from small volumes (25 ml.) with those of the same strains grown in large volumes of medium (500 ml.). The value of carbon dioxide was investigated by growing cultures (25 ml. medium), with and without increased carbon dioxide tension. The presence of a precursor for lipase, as observed by Elliott (1945, 1950) for streptococcal proteinase, was investigated by decreasing the incubation time to 24 and 48 hr. Cultures were prepared in triplicate, and in two sets the pH value of the medium was varied by adding glucose in final concentrations of 0·5 and 1·0 %, the buffered peptone being omitted. The filtrates of these cultures were divided into two parts and 0·1 % sodium mercaptoacetate (thioglycollate) added to one portion of each. Each portion was then divided again and one half stored at 4° and the other at room temperature for 1 week. All were then retested for lipolytic activity. The heat stability of staphylococcal lipase was measured by holding filtrates at different temperatures in a water-bath for varying periods of time. The activity was then assayed on N.B.S.A. as described above. The temperatures used were 56, 70, 85 and 100°. The stability on storage was measured by keeping filtrates at 4° for 9 months and assaying the lipolytic activity every few weeks.

Production of anti-lipolytic antibody in rabbits. Before inoculation all rabbits were bled from the ear and their sera examined for naturally occurring anti-lipolytic antibody. Two methods of immunization were used. (*a*) Living cultures of albus strains; 24 hr. digest broth cultures diluted 1/10 were injected intravenously into rabbits in increasing doses (0·1–0·4 ml.) on successive days, followed by 4 days of rest. This course was repeated a week later and in two subsequent weeks when undiluted cultures were used. Samples of serum were taken 4 days after the last injection. Three rabbits were used for each strain. (*b*) Culture filtrates of aureus and albus strains; filtrates were inoculated intravenously on 3 successive days followed by 4 days of rest, for 4 weeks, doses being increased gradually from 0·1 to 1·0 ml. The toxin present in filtrates of aureus strains was converted to toxoid by the method of Dolman & Kitching (1935).

Titration of immune rabbit serum for anti-lipolytic activity. Serial dilutions of the homologous filtrate were dropped on to N.B.S.A. plates and drops of immune serum, undiluted and diluted 1/5 to 1/40, were added immediately before the filtrate had been absorbed into the medium. The filtrate and serum drops were mixed and after absorption into the agar, the plates were incubated as for titration of filtrates. Duplicate plates were used in all titrations of anti-lipolytic activity. Two controls were used, corresponding dilutions of the filtrate alone, and filtrate mixed with undiluted normal rabbit serum (Pl. 1, fig. 3).

RESULTS

Satisfactory results in the isolation of lipolytic staphylococci were obtained using N.F.A. medium, which was preferable to N.B.S.A. for cultures because it eliminated the 'false positive' results described by Turner (1929). N.B.S.A., however, was found preferable to N.F.A. for culture filtrates of lipolytic strains as the dye appeared to sharpen the definition of the zones.

Compared with albus strains of staphylococci more aureus strains were found to produce diffusible lipase (Table 1). When other biochemical activities (sugar fermentations, production of coagulase, haemolysis on blood agar) were investigated, it was found that in general lipolytic strains tended to be more active biochemically than non-lipolytic ones (Table 2).

Lipase was demonstrable in culture filtrates only when staphylococci were grown in conditions known to be required for the formation of other toxins, notably the α, β and γ haemolysins, although the presence of these toxins in the filtrates prepared was not investigated.

Staphylococcal lipase proved to be relatively stable in culture filtrates *c.* pH 8, filtrates of aureus strains being slightly more stable than those from albus strains. Approximately half of the activity was lost by heating for 1 hr. at 70° or for 45 min. at 85°; boiling for 40–50 min. destroyed the activity completely. With the majority of strains, only a very gradual loss of lipolytic activity from the culture filtrates was observed during storage at 4° for 9 months; this was apparent more by loss of differentiation of zones than by a decrease in their diameter.

Table 1. *Staphylococci tested for the production of diffusible lipase*

Species	No. of strains tested	No. of lipolytic strains		No. of non-lipolytic strains	
		Coagulase positive	Coagulase negative	Coagulase positive	Coagulase negative
S. aureus	48	39	2	5	2
S. albus	38	11	18	3	6
S. citreus	2	1	0	0	1

Table 2. *Comparison between the production of diffusible lipase by staphylococci and some other biochemical activities*

	No. of strains tested	No. of sugars fermented	No. of strains producing coagulase	No. of strains liquefying gelatin	No. of strains haemolytic on blood-agar
Lipolytic strains	42	6/12	38	33	32
Non-lipolytic strains	8	Fewer than 6/12	1	0	2

Table 3. *Anti-lipolytic activity of normal and immune rabbit serum against the homologous filtrate*

Filtrate	Serum	Dilution of serum	Maximum dilution of filtrate showing lipolysis
S. aureus W3	0	—	1/512
	Normal	1/1	1/512
	Immune W3	1/1	1/4
	Immune W3	1/5	1/16
	Immune W3	1/10	1/32
	Immune W3	1/20	1/128
	Immune W3	1/40	1/256
S. albus 8	0	—	1/256
	Normal	1/1	1/256
	Immune 8	1/1	1/4
	Immune 8	1/5	1/32
	Immune 8	1/10	1/64
	Immune 8	1/20	1/128
	Immune 8	1/40	1/128

Table 4. *Anti-lipolytic activity of four rabbit antisera against homologous and heterologous filtrates*

	Titre of filtrate after mixing with undiluted anti-lipolytic serum							
	Filtrates from aureus strains				Filtrates from albus strains			
Antiserum	W3 (1/256)	W4 (1/128)	M3 (1/64)	M5 (1/128)	8 (1/128)	16 (1/32)	20 (1/32)	39 (1/64)
W3	U	—	—	—	U	1/4	—	—
W4	1/2	—	U	U	—	1/2	1/2	U
8	1/2	—	1/2	U	—	U	1/4	1/2
39	1/8	—	1/4	U	—	1/2	U	—

Initial titre of culture filtrate in brackets; —, lipolytic activity of filtrate neutralized completely; U, lipolytic activity of filtrate neutralized except in the undiluted state.

Antibody was formed readily in rabbits, none of the strains studied failing to stimulate antibody production. No difference was observed in the antigenicity of albus strains and culture filtrates of the same strains; all antisera neutralized the lipolytic activity of the homologous filtrate and also that of a wide range of heterologous filtrates, in many cases to a comparable degree. Examples of the titrations are shown in Tables 3 and 4. The maximal neutralization was given by undiluted antiserum against a range of dilutions of the filtrate; the most potent antisera neutralized 250 times the lipolytic titre of the filtrate while dilutions of 1/5 and 1/10 neutralized 60 and 16 times the lipolytic titre of the filtrate, respectively.

No naturally occurring anti-lipolytic antibody was found; the sera were not examined for the presence of any other staphylococcal antitoxin.

DISCUSSION

Many workers have studied the production of lipase by bacteria and obtained conflicting results. Some used techniques in which only diffusible lipase (exolipase) was demonstrated, while others used methods in which an endolipase, liberated from cells by autolysis or chemical extraction, was being detected as well as exolipase. The production of endolipase may in part explain the so-called 'false positive' results (Turner, 1929) found in the present work when certain media were used. In this reaction the blue colour is most apparent in the centre of the colony, or in streak cultures along the centre of the streak, where presumably the oldest cells are to be found, and autolysis is most likely to occur. This phenomenon may also in part be due to adsorption of the dye by the bacterial cells.

Boiling for at least 40 min. was required to destroy the lipase activity completely. It is difficult to compare this result with that of MacFarlane & Knight (1941), who investigated the heat-stability of lecithinase, because experimental conditions were different. It would seem, however, that staphylococcal lipase is relatively stable compared with the majority of enzymes, which are inactivated at 65°, and it belongs rather to the group which includes crystalline trypsin which, in acid solution, is not wholly destroyed by heating at 100° (Sumner & Myrbäck, 1950).

The majority of the culture filtrates examined showed only a very slow decline in lipolytic activity during storage at 4° for 9 months during which time less than 20% of their lipolytic activity was lost. Those which ceased to be lipolytic had shown a low initial titre, and no detectable lipolytic activity remained in them after storage for 4 weeks.

The cross-neutralization tests carried out with staphylococcal culture filtrates and antisera gave consistent results, and the evidence obtained suggested that the lipases of staphylococci are antigenically homogeneous.

Staphylococcal lipase differed from some of the other bacterial enzymes such as catalase (Campbell & Fourt, 1939) which have been used as antigens in that neutralization by heterologous antisera was almost, if not entirely, as marked as with homologous antiserum.

A search of published literature failed to provide any other recorded experiments in which anti-lipase has been produced as a result of animal injection with culture filtrates containing diffusible lipase. One recorded report was found, however (Gillissen, 1953), in which an anti-lipase was produced by Sartory, Meyer and Lange (1947) with whole cultures of tubercle bacilli. The neutralizing activity of that anti-lipolytic antibody against lipases other than those of tubercle bacilli was investigated with negative results. In the present work no tests were made against any lipase except that of staphylococci.

The author wishes to express her thanks to Prof. Lord Stamp for supervising this work; to Mr E. V. Willmott, F.R.P.S., for taking the photographs, and to the Examiners of London University for permission to publish part of her Thesis.

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EXPLANATION OF PLATE

Fig. 1. A lipolytic strain of *Staphylococcus aureus* showing a surrounding area of clearing, on a 48 hr. N.F.A. plate. On the other half of the plate (right-hand side) is a strain of *Escherichia coli* which is not lipolytic and has produced no change in the medium.

Fig. 2. Zones of activity from undiluted culture filtrates of four lipolytic strains of *S. aureus* on N.B.S. agar. The central cylinder contained a heated filtrate, which produced no change in the medium. (The cylinders were removed before taking the photographs.)

Fig. 3. The lipolytic activity of a culture filtrate of *S. aureus* diluted serially up to 1/512: upper left quadrant, filtrate alone; upper right quadrant, filtrate mixed with normal rabbit serum; lower left quadrant, filtrate mixed with immune rabbit serum, undiluted; lower right quadrant, filtrate mixed with immune rabbit serum, diluted 1/10.

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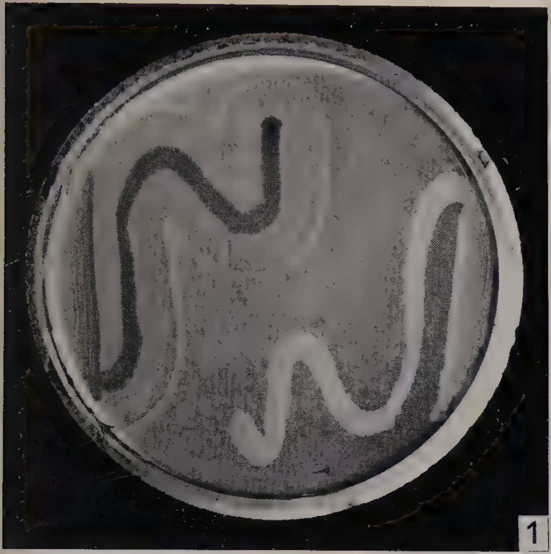
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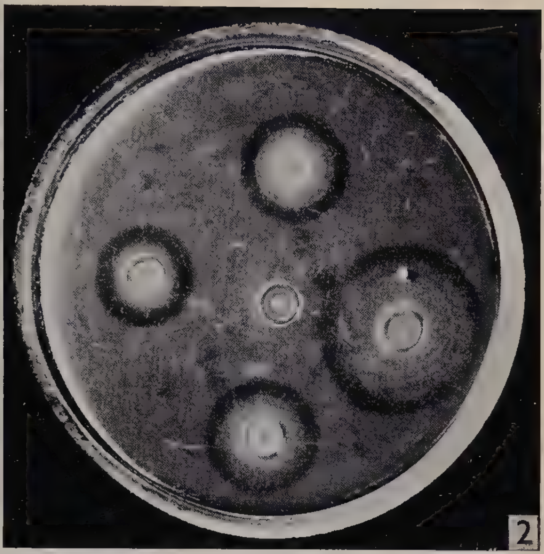
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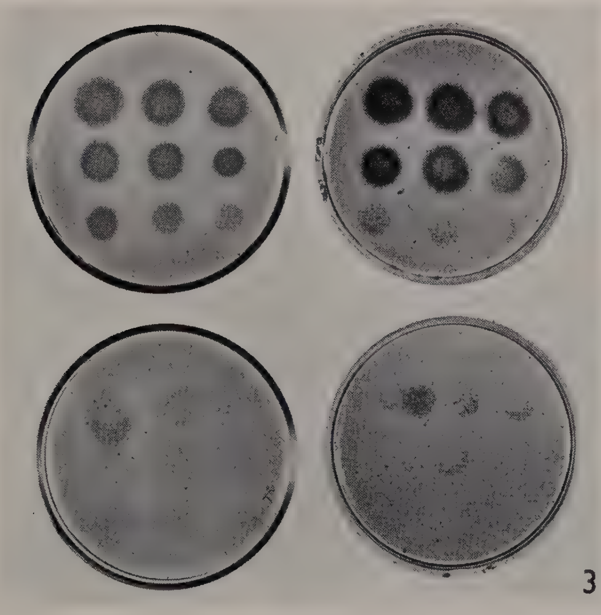
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M. Davies—Diffusible lipase of staphylococci

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The Deoxyribonuclease of Typhoid Bacilli and its Effect on Typhoid Bacteriophage Nucleic Acid

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SUMMARY: Deoxyribonuclease (DNase) was found in extracts of a strain of *Salmonella typhi.* Its action on herring sperm deoxyribonucleic acid (DNA) is catalysed by magnesium ions. Calcium ions, although inactive alone, increase the magnesium-catalysed activity of the enzyme. A highly polymerized specimen of phage DNA was prepared by treatment of the purified phage with strong urea solution to dissolve the protein coat. This nucleic acid is degraded by the typhoid DNase in the presence of magnesium. As with herring sperm DNA, calcium is inactive alone but increases the action of magnesium. Calcium is required for multiplication of the phage in the host bacterium, but the hypothesis that the calcium is required to catalyse the breakdown of phage DNA by bacterial DNase was not upheld.

Interest in the relation between deoxyribonuclease (DNase) and bacteriophage derives from their common requirement for bivalent metals and the high content of deoxyribonucleic acid (DNA) in bacteriophage. Mammalian DNase is catalysed by magnesium ions (McCarty, 1946) and certain phages, notably coli phage T5 (Adams, 1949; Kay, 1952) and a typhoid phage (Fildes, Kay & Joklik, 1952) require bivalent metals for their multiplication. Calcium ions are highly specific for the typhoid phage and are the most active of a small group of metal ions for phage T5. In the typhoid phage system the absence of calcium causes the majority of the phage particles in a mixture of phage and bacteria to be inactivated. This inactivation is a consequence of adsorption to the cells and does not take place when the phage alone is suspended in calcium-free medium.

If the findings of Hershey & Chase (1952) with coli phage T2 are applicable to the typhoid phage, then the next stage after adsorption has taken place is the transfer of nucleic acid from the phage to the bacterium, the protein envelope of the phage taking no further part in the multiplication of the virus. The nucleic acid of the parent phage is not all transferred to the progeny, the amount varying from 23 to 62 % in different experiments (French, Graham, Lesley & van Rooyen, 1952). Hershey (1953) reported that only 40 % of the parental phosphorus was found in the progeny. The transfer of a small but specific part of the parent phage nucleic acid to the progeny has not been ruled out but it has been suggested by Kozloff (1952) in the case of coliphage T6 that most of the parent virus material found in the progeny was due to the use of breakdown products of the parent virus for the synthesis of new virus nucleoprotein. This suggests, although direct evidence is lacking, that the breakdown of phage DNA might be a necessary stage in the multiplication of the virus. If this were so then DNase would probably take part in the degradation. The phages which require calcium for their multiplication may possess nucleic acids of a different type from those of other phages in that the action of DNase is catalysed by calcium instead of magnesium. If this were so then the action of calcium could be explained as that of a catalyst in the breakdown of the phage nucleic acid into polynucleotides prior to their taking part in the production of new phage particles. In the absence of calcium the phage DNA could not be broken down and might even be prevented by the large size of its molecules from entering the cell. From this hypothesis it was expected that the host bacteria would contain DNase and that its action on phage DNA would be different from its action on a non-viral DNA. The work to be described in this paper comprises (*a*) the examination of a strain of *Salmonella typhi* for DNase, (*b*) the preparation of typhoid phage DNA, and (*c*) the study of the effect of calcium and magnesium on the interaction of the two.

MATERIALS AND METHODS

Bacteria. The strain of *Salmonella typhi* described by Kay & Fildes (1950) was used. This is a 'rough' mutant derived from NCTC 3390. Cultures were grown in a glucose-ammonia medium as described by Kay (1952).

Bacteriophage. The phage was the same strain as that used by Kay & Fildes (1950). It was prepared and purified by the same technique as that described for coli phage T5 (Kay, 1952). Dr A. Felix has examined this phage and states that it is indistinguishable from his standard O phages No. 1, 2 and 3.

Deoxypentose nucleic acid. This was prepared from herring sperm by the method of Daly, Allfrey & Mirsky (1950).

Estimation of deoxyribonuclease activity. The spectrophotometric method of Kunitz (1950) was used except that acetate buffer was replaced by phosphate buffer at pH 7·6. Undegraded nucleic acid was precipitated from the samples by the addition of $0{\cdot}25\,\text{N-}H_2SO_4$ and the material rendered acid-soluble by enzyme action was estimated by its absorption at 260 mμ. Measurements were made in a Hilger 'Uvispek' absorptiometer.

RESULTS

Deoxyribonuclease in typhoid bacilli

Extracellular DNase production by this organism was tested for by spreading the bacteria on the surface of nutrient agar impregnated with 0·3 % (w/v) of DNA and incubating overnight at 37°. When the plates were flooded with a solution of mercuric chloride in HCl, a reagent which precipitates the undegraded DNA in the agar causing a turbidity to appear, it was seen that the bacteria did not produce a detectable extracellular enzyme as the DNA was precipitated right up to the colonies. A control plate spread with a strain of *Bacillus subtilis* known to produce DNase showed clear areas round the colonies.

Intracellular DNase was tested for by disintegrating the cells, incubating the product with a solution of DNA and measuring the release of acid soluble degradation products of the nucleic acid. The cells from an overnight culture in 100 ml. chemically-defined medium were harvested by centrifugation, suspended in 10 ml. of 0·01 M-phosphate buffer at pH 7·6, and crushed by vibration with 'ballotini' beads in a Mickle shaker. After 15 min. shaking the beads were removed by decantation and the supernatant containing both soluble and insoluble cell material was tested as follows. To 5 ml. of a solution of DNA in water (1 mg./ml.) was added 1 ml. 0·1 M-magnesium sulphate and 0·1 ml. crushed cell suspension in buffer (pH 7·6), the final concentration of which was 0·00016 M. The concentration of the crushed cell material was 200 μg./ml. The mixture was incubated at 37°. Samples (1 ml.) were withdrawn at intervals and added to 4 ml. ice-cold 0·25 N-sulphuric acid. After standing for 30 min. the precipitates were removed by centrifugation and the ultraviolet absorption of the supernatants at 260 mμ. was measured. A suspension of whole cells of the same concentration as that used to prepare the crushed cells was also tested and controls of DNA alone and crushed cells alone were included. The results are shown in Fig. 1. DNA alone was not degraded but the whole cells had a slight effect. Presumably this activity was too small to show in the impregnated agar test. The crushed cells, on the other hand, had a marked effect which indicated that the bacteria possessed an intracellular DNase.

It was now of interest to determine whether the enzyme was in solution or attached to the bacterial debris. The crushed cells were centrifuged at 4000 $\boldsymbol{g}$ for 10 min. and the opalescent supernatant was decanted. The sediment was taken up in phosphate buffer and it and the supernatant were tested for DNase activity by the method described above. The results which are given in Fig. 2 show that the activity of the supernatant is almost equal to that of the crushed cells, and that the enzyme is either in solution or present in the form of very small particles unsedimentable at 4000 $\boldsymbol{g}$. This supernatant was used in subsequent experiments and is referred to as 'typhoid DNase'.

The ordinate in Figs. 1 and 2 is the absorption at 260 mμ. of the acid soluble degradation products of the DNA. The initial reading of about 0·5 is due to the presence in the sample of DNA of a small amount of acid-soluble material, probably produced by the partial degradation of the DNA during its preparation.

The effect of magnesium and calcium on typhoid deoxyribonuclease

Calcium and magnesium were tested as activators of typhoid DNase by addition of solutions of the chloride and sulphate respectively to mixtures of the enzyme preparation and DNA. Samples were taken from the incubated mixture and the amount of degradation measured as in previous experiments. Calcium was found to have no effect, the enzyme remaining inactive at concentrations of this element between 0·0005 and 0·01 M. Magnesium, on the other hand, showed an activity increasing with concentration (Fig. 3). However, when magnesium and calcium were tested together it was found that the calcium increased the activity of the magnesium. A typical example of this effect is given in Fig. 4 in which the addition of 0·003 M-calcium to 0·003 M-magnesium increased the rate of degradation of DNA by 75 %, whereas calcium alone had no effect at all.

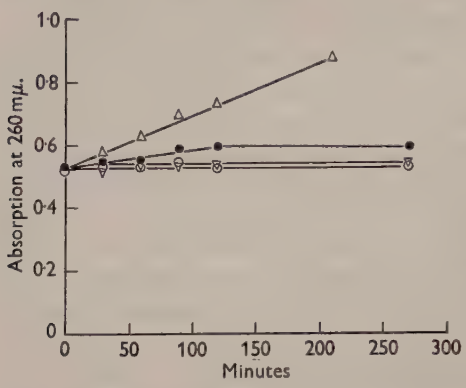

Fig. 1

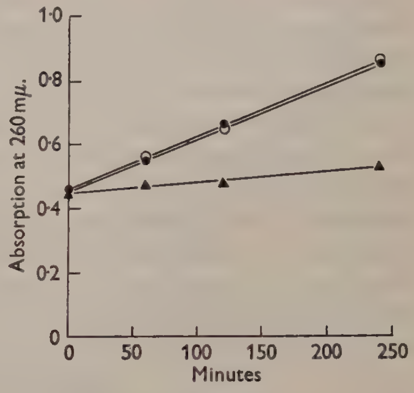

Fig. 2

Fig. 1. The action of whole and crushed *Salmonella typhi* on DNA (800 μg./ml.) at pH 7·6 and 37°. The cell concentration whole and crushed was 200 μg./ml. ○—○, DNA alone; ●—●, whole cells + DNA; △—△, crushed cells + DNA; ∇—∇, crushed cells alone.

Fig. 2. The action of the supernatant and sediment from centrifuged crushed *Sal. typhi* on DNA (800 μg./ml.) at pH 7·6 and 37°. The crushed cell concentration was 200 μg./ml. ○—○, crushed cells + DNA; ●—●, supernatant from crushed cells + DNA; ▲—▲, sediment from crushed cells + DNA.

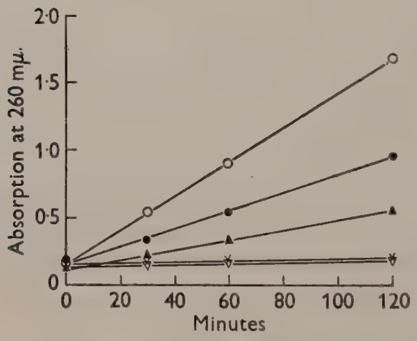

Fig. 3

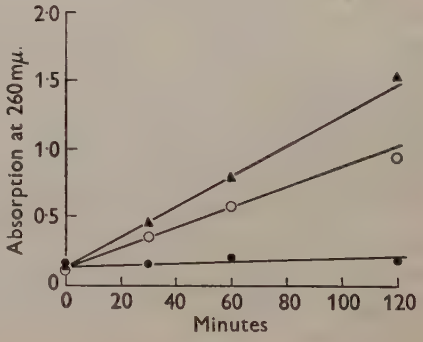

Fig. 4

Fig. 3. The effect of magnesium and calcium on the action of typhoid DNase on DNA (800 μg./ml.) at pH 7·6 and 37°. ○—○, 0·016 M-magnesium; ●—●, 0·0033 M-magnesium; ▲—▲, 0·0008 M-magnesium; ∇—∇, no magnesium; ×—×, 0·01 M-calcium.

Fig. 4. The effect of calcium on the action of typhoid DNase on DNA (800 μg./ml.) activated by magnesium at pH 7·6 and 37°. ●—●, 0·0033 M-calcium; ○—○, 0·0033 M-magnesium; ▲—▲, 0·0033 M-calcium + 0·0033 M-magnesium.

Typhoid deoxyribonuclease and typhoid phage deoxypentose nucleic acid

DNase is unable to attack phage DNA while the phage is intact (Hershey & Chase, 1952). This is probably due to the fact that the nucleic acid is enclosed by a protein coat impermeable to the enzyme. Therefore in order to study the effect of DNase on phage DNA the protein coat must be removed and the DNA exposed. As it is necessary to prepare the DNA in as highly polymerized a form as possible the procedure used must be a mild one. Cohen (1947) described the use of strong urea solution to liberate the nucleic acid of coliphages T2 and T4. This method, in modified form, was tried on the typhoid phage. Two litres of lysate containing 3×10^{10} phage particles/ml. were prepared and the phage separated by differential centrifugation. The phage was resuspended in 0·01 M-phosphate buffer and again sedimented at high speed. The sediment was taken up in a mixture of 5 ml. 50 % (w/v) solution of urea in water and 1 ml. 0·1 M-glycine + NaOH buffer (pH 9·6). The mixture was stirred vigorously and a clear viscous solution was formed. Protein was removed by repeated shaking with chloroform + amyl alcohol mixture and the nucleic acid precipitated by pouring into 100 ml. 95 % ethanol. The fibrous precipitate was washed in 95 % ethanol and dried with absolute ethanol and ether. The product (6·6 mg.) when dissolved in water (1 mg./ml.) gave a weak gel. Its ultraviolet absorption spectrum showed a peak at 260 mμ. typical of nucleic acid, and paper chromatography in *iso*propanol/HCl of a formic acid hydrolysate showed the presence of adenine, guanine, thymine and 'cytosine'. It is not known whether the spot visible in the cytosine position was cytosine or 5-hydroxymethylcytosine, which is present in the T-even coli phages, as both these substances migrate to the same place in this solvent (Wyatt & Cohen, 1952). No uracil was found; the purines and pyrimidines were those typical of deoxyribonucleic acid.

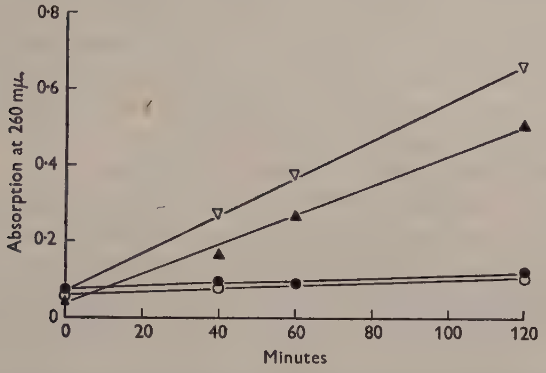

Fig. 5. The effect of calcium and magnesium on the breakdown of phage DNA (800 μg./ml.) by typhoid DNase at pH 7·6 and 37°. ●—●, 0·0033 M-calcium; ▲—▲, 0·0033 M-magnesium; ▽—▽, 0·0033 M-calcium + 0·0033 M-magnesium; ○—○, no addition.

The effect of typhoid DNase on phage DNA was examined in the same manner as on the herring sperm DNA, except that the volume of the reactants was reduced to conserve material. The total volume of the mixed reactants was 1·0 ml. and samples of 0·1 ml. were taken. The ultraviolet absorption of the acid soluble material was measured in the micro-cells of the Uvispek instrument. The results of an experiment to test the effect of calcium and magnesium are given in Fig. 5. The degradation of phage DNA was catalysed by magnesium but not by calcium, and as in the case of herring sperm DNA calcium increased the activation caused by magnesium, although to a lesser extent.

DISCUSSION

Several species of bacteria which produce DNase have been described, for example various clostridia and streptococci, but in all cases the enzyme has been secreted into the medium. The particular strain of *Salmonella typhi* used in the present experiments produced very little extracellular enzyme although one is present, as expected, in the cell contents. The action of this enzyme, in the form of a crude extract, on herring sperm DNA is catalysed by magnesium ions as is the action of DNase of mammalian origin (McCarty, 1946). Calcium is not an activator but it does increase the action of magnesium. The breakdown of DNA derived from bacteriophage is also catalysed by magnesium. Again calcium, which is inactive alone, increases the rate of the magnesium catalysed reaction. This effect could be due to the displacement of magnesium by calcium from combination with sites which are not essential for enzymic action. These sites may exist on protein impurities in the crude enzyme preparation. The released magnesium could then combine with the substrate-enzyme complex in such a way as to cause activation.

The experiments described in this paper do not disclose any property of the nucleic acid which could account for the peculiar calcium requirement of the phage. On the contrary, the remarkably similar reactions of phage DNA and herring sperm DNA with DNase show that these two preparations, although from widely different sources, possess some similarity of structure.

My thanks are due to Sir Paul Fildes, F.R.S., for his interest in the work and his valuable criticism, and to the Medical Research Council for a personal grant.

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Nuclear Division and the Life Cycle in a *Streptomyces* sp.

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SUMMARY: The life cycle of a *Streptomyces* sp. was studied by the Robinow HCl-Giemsa method of nuclear staining, and is described in the following manner: (1) initial nuclear division phase; (2) primary mycelium; (3) secondary mycelium (including aerial); (4) the formation of spores. It was observed that the method of nuclear division in the ungerminated spores closely resembled mitosis of certain higher organisms and lower plants, by the appearance of two terminal bodies whose behaviour was similar to mitotic centrosomes. The primary mycelium developed after the initial nuclear division phase and produced side branches, then later gave rise to single swellings in the hyphae. These swellings grew to form large round cells, each of which contained many nuclei. It was from these cells that the secondary mycelium developed, a part of which became aerial and terminated in the form of chains of uninucleate spores.

Recent developments in the cytology of bacteria have brought forth the publication of many photomicrographs in which nuclear configurations have been interpreted as phases in mitosis (Delamater, 1951; Delamater & Woodburn, 1952; Beutner, 1953). But the demonstration of a mitotic spindle in bacteria has been seriously questioned on the grounds that it is possible to confuse artifacts with mitotic spindles (Bisset, 1953). It is the purpose of this paper to present observations of a nuclear division that closely resembles mitosis in higher organisms and of the life cycle of a *Streptomyces* sp. whose taxonomic position links the bacteria with the moulds (Henrici, 1947).

In the course of my work on the genetic effects of radiations on a *Streptomyces* sp., a cytological study was undertaken to determine the time of nuclear division in the germinating spores, and to observe whether the secondary mycelium arose from distinctive 'initial' cells similar to those described in actinomyces by Klieneberger-Nobel (1947). In the streptomyces examined it was observed that the first nuclear division was preceded by the formation of two terminal bodies arranged similarly to those already demonstrated in certain bacteria by Delamater (1951) and who interpreted his observations as a mitotic division mechanism. But in the streptomyces spores the terminal bodies were more readily distinguishable. At a certain stage of growth in the primary mycelium the initial cells were seen to develop from swellings in the hyphae, and not from 'nests' of tangled filaments as was observed by Klieneberger-Nobel (1947) in actinomyces.

MATERIAL AND METHODS

An organism, a *Streptomyces* sp. (laboratory strain T12) isolated from the soil by Dr J. W. Rouatt was used. It produces colonies consisting of a primary mycelium with deep orange colour, and a fluffy layer of paler orange secondary or aerial mycelium.

Spores were grown on M12 agar (asparagine, Bacto peptone, dipotassium phosphate 0·5 g. each; glucose 10 g.; Bacto agar 15 g.; and tap water 1000 ml.) for the examination of early stages of colony development; but for examining the later stages of growth liquid cultures were studied since the hyphae began to branch and burrow into agar. For the examination of the early stages an agar block was cut from a 7-day stock culture on M12 medium, and pressed against a new agar plate, moving it over the surface to form an inoculum of diminishing density; the plate was then incubated at 28°. A number of types of liquid culture were tried, but the best results were obtained by incubating the spores in M12 liquid medium between cover-glass and slide. In this manner, micro-colonies grew on the borderline of the air and medium, and were kept flat by the surface of the cover-glass. Mature colony impressions were also made from liquid and solid cultures.

The Robinow (1945) method of nuclear staining was used. This consisted of fixing agar blocks of spores for 2 min. in the vapour of 2 % osmic acid, transferring the spores to albuminized cover-glasses by impression, immersion in mercuric chloride (saturated) for 4 min., hydrolysing in N-HCl at 60° for 8 min., and then staining with Giemsa stain (Gurr's R66, 20 drops in 10 ml. phosphate buffer, pH 7·0) for 10–20 min. Permanent mounts were made in Abopon (Delaporte, 1948–9; obtained from Glyco Products Co. Inc., 26 Court St, Brooklyn, N.Y., U.S.A.).

Photomicrographs were made using the following: (1) × 95 Leitz fluorite oil-immersion lens; (2) × 10 hyperplane eyepiece; (3) aplanatic condenser; (4) Köhler illumination; (5) yellow-green filter for contrast; (6) a Leica 35 mm. camera. This optical system gave a flat field without introducing chromatic aberration.

RESULTS

The streptomyces are a saprophytic group of the Actinomycetales growing in the form of a much-branched mycelium which terminates growth by forming chains of spores on aerial hyphae (Waksman & Henrici, 1943). This genus has been divided on the basis of the structure of its sporulating hyphae (*Bergey's Manual*, 1948), but apart from this there is little literature about a regular morphological description of the actinomycetes. Klieneberger-Nobel (1947) described the life cycle of spore-bearing actinomyces under the following headings: (1) primary mycelium; (2) origin of initial cells; (3) secondary mycelium; (4) formation of spores. In the present investigation the species of streptomyces examined will be similarly described but with an additional 'initial nuclear division phase'.

Initial nuclear division phase

With the *Streptomyces* sp. studied here, between the time of implanting the spores on agar and germination (3·5 hr.), the form of the spore (Pl. 1, fig. 1) changed from oval to pear-shaped with the blue-coloured nucleus at the broad end of the cell, and a red-coloured structure at the narrow end (Pl. 1, fig. 2). This clearly defined structure will be referred to as the terminal body.

On further incubation a second terminal body became visible in the broad end of the cell, and then travelled around from the nucleus to alter the shape of the cell so as to form two narrow ends each with a terminal body, and a broad central portion in which the nucleus remained (Pl. 1, figs. 3, 4). At this stage the cell was elongated to 3 or 4 times its previous length, and the terminal bodies had the appearance of being strongly repelled by one another and by the nucleus.

The terminal bodies provided a number of parallels with the centrosomes (Sharp, 1943) of higher organisms in that: (*a*) before nuclear division occurred a single terminal body appeared followed by a second which may have been formed by division of the first; (*b*) the terminal bodies diverged until they reached the opposite sides of the nucleus; (*c*) shortly after this formation the nucleus divided.

With the relation of likeness between the nuclear division of the streptomyces spore and mitotic division of higher organisms the author has defined the nuclear forms observed in the following manner; resting cells—cells with no terminal bodies; prophase cells—cells with only one terminal body; metaphase cells—two terminal bodies, one at either side of the nucleus, the nucleus not visibly divided; anaphase—nucleus visibly divided.

Table 1. *Nuclear changes in streptomyces spores*

Time of observation (hr.)	Resting cells* (%)	Prophase (%)	Metaphase (%)	Anaphase (%)	No. of cells counted
0	100	—	—	—	500
1	85·2	14·1	0·7	—	141
2	55·9	41·2	2·0	—	143
3	4·7	65·8	21·1	2·9	170

* The phases in nuclear division have been defined above.

0·9 % of the cells at 2 hr. and 5·5 % of the cells at 3 hr. were not clearly scorable, and have not been included in the above percentages.

The nucleus then dividided into two and one end of the cell lost its pointed appearance (Pl. 1, figs. 5, 6), while the other end formed the germination tube into which one daughter nucleus entered and underwent further division, presumably in the same manner, though this could not be determined precisely because the hyphae stained so faintly that the contents were obscured. Pl. 1, fig. 7, shows the sequence of changes that occur during the first 3·5 hr. of incubation, and Table 1 gives the proportions on the different phases at different times. Pl. 1, figs. 8, 9, show a comparison of the cell morphology at 2 and 3 hr.

Primary mycelium

When the streptomyces spores were incubated for 5·5 hr. fine threads were seen branching from the sides of the main hyphae (Pl. 2, fig. 10), which on solid medium burrowed into the agar. At this time sixteen to thirty nuclei might be seen, and at least 90 % of the spores had produced one hypha, and about 5 %, two hyphae. Shortly after this time nearly all the spores developed a second hypha. A terminal body was seen to reappear in the original spore, followed by division of the nucleus and the sprouting of the new hypha from the opposite end of the cell. One of the two daughter nuclei migrated in to this new hypha and underwent further division, while the spore case was left with a single nucleus. The development of more than two hyphae from the one was spore not observed in the T12 strain of streptomyces used.

After 16–20 hr. of incubation of slide cultures, each strand of mycelium developed a swelling which contained many nuclei (Pl. 2, figs. 11, 12). These swellings grew into large round cells ('initial' cells) of about 4 times the diameter of the spore cell, and some of them were seen attached to the hyphae by short stalks (Pl. 2, fig. 13). After this stage the initial cells presumably broke away from the hyphae, and from them the secondary mycelium developed.

Secondary mycelium

Each strand of the new mycelium seemed to develop from an aggregation of a nucleus and cytoplasm of the initial cell. The strands grew in a radial pattern, and a number of nuclei with no obvious cell wall around them were seen at the centre (Pl. 2, fig. 15). These new hyphae grew in the form of long slender threads which produced fewer side branches than the primary mycelium. A striking feature of the secondary mycelium was that some of the strands were composed of deeply staining cylinders which had a length about 10 times the diameter of a spore; there were non-stainable gaps between the ends of these cylinders (Pl. 2, fig. 17). Other strands were seen as one continuous deeply stained cylinder. The secondary mycelium with these types of strands formed the matted surface of the colony; the strands which became aerial developed stained regions of shorter and more uniform length, and gave rise to the formation of chains of spore nuclei.

Spore formation

It seemed apparent that the part of the secondary mycelium which became aerial changed from the deeply stained cylindrical forms to forms in which there were segments which stained less densely as though the nuclear substance were condensing (Pl. 2, fig. 16, right arrow). The appearance of these areas marked the beginning of development of a visible chain of spore nuclei (Pl. 2, fig. 16, left arrow), which were later seen in the forms of: rectangular blocks and bodies that were either round or square (Pl. 2, fig. 18). Before the formation of the spores these irregular shapes changed to round nuclei of constant size, uniformly spaced along the strand. Shortly after this stage it was observed that single spores and short chains of spores broke off readily; from this it was inferred that transverse septa between the nuclei had developed.

The author wishes to thank Dr H. B. Newcombe for his criticism of the manuscript, and Mr N. R. Vincent for the preparation of the photographs.

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EXPLANATION OF PLATES

Streptomyces sp., stained by HCl-Giemsa

Plate 1

Figs. 1–7, ×2360; figs. 8, 9, ×1180

Fig. 1. Spores in the resting stage; nucleus located centrally and terminal bodies absent.

Fig. 2. Spores after 2 hr. incubation on agar; terminal bodies visible at the narrow ends of the cells (uppermost part of the photograph).

Fig. 3. Elongated cells with the nucleus remaining at the centre and a terminal body at each end; note cells left of centre and at bottom right, the nucleus has divided and the terminal bodies are disappearing.

Fig. 4. Similar to fig. 3. Note the cell at the extreme left; early germination, terminal bodies absent.

Figs. 5, 6. Cells showing the two halves of the nucleus separating.

Fig. 7. The changes occurring in the cell from the resting stage until germination; 3·5 hr.

Figs. 8, 9. Comparison of cells after 2 and 3 hr. incubation.

Plate 2

Figs. 10–18, ×2360

Fig. 10. Primary hypha with side branches of mycelium; 7·5 hr. incubation.

Figs. 11, 12. Swellings which develop into 'initial' cells in the primary hyphae; note nuclear shape within the swellings, and the comparison of the nuclear structure of the hyphae to the hypha in fig. 10.

Fig. 13. Initial cell at the end of a short stem of mycelium.

Fig. 14. Ruptured initial cell showing nuclear contents.

Fig. 15. Development of secondary mycelium from initial cell.

Fig. 16. Transition of secondary mycelium to aerial mycelium; arrow at the right pointing to areas which stain less densely; the arrow at the left indicating the formation of a chain of nuclei.

Fig. 17. Secondary mycelium with long deeply stained cylinders showing non-staining areas between the cylinders.

Fig. 18. A strand of aerial mycelium showing the nuclei changing from irregular shapes to a uniform pattern.

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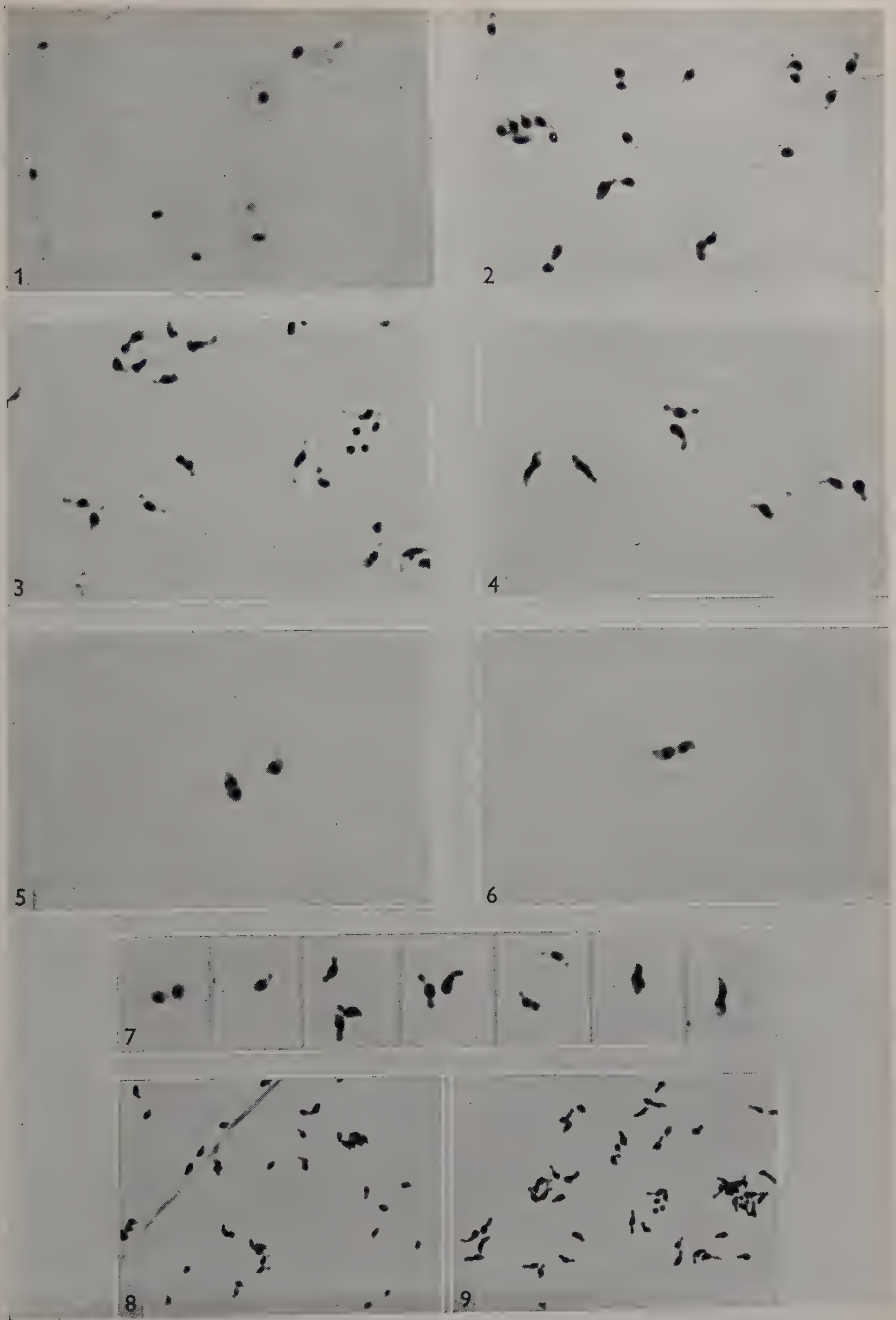
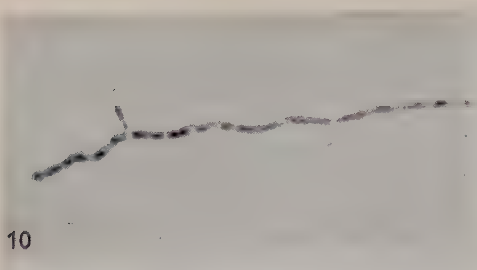


J. F. McGregor—Life cycle in a *Streptomyces* sp. Plate 1



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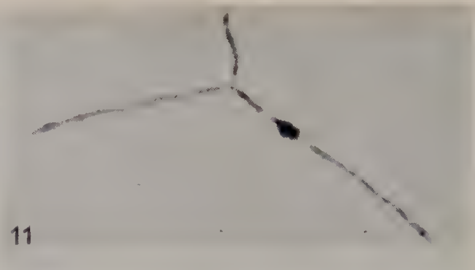

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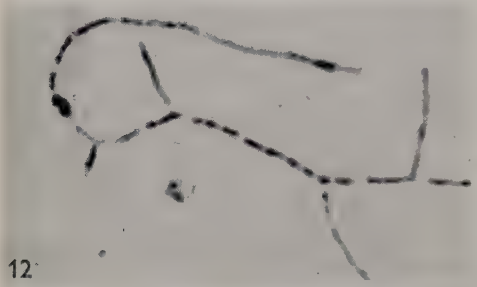

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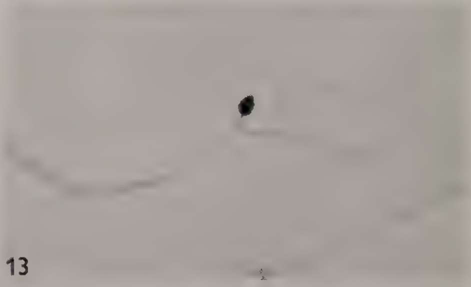

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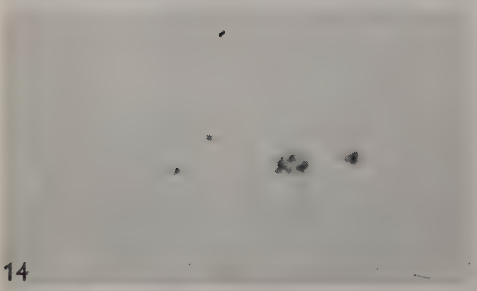

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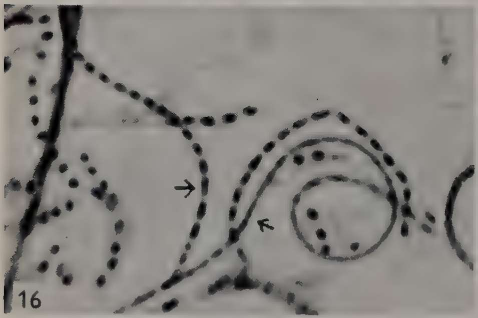

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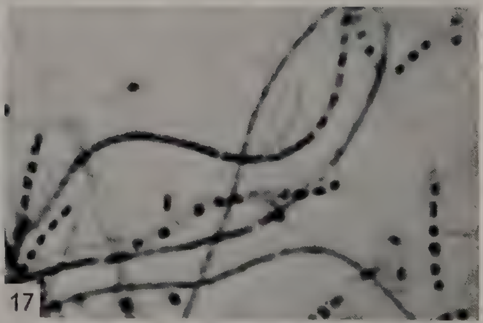

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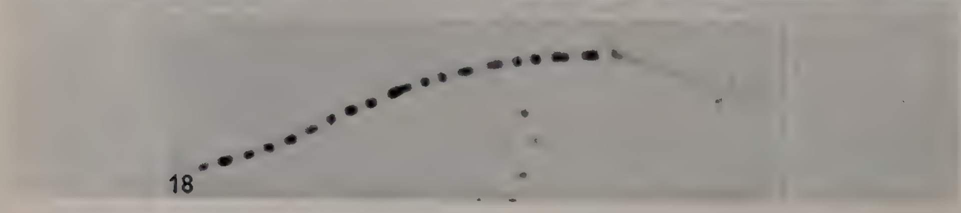

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J. F. McGregor—Life cycle in a *Streptomyces* sp. Plate 2

Beale, G. H. & Schneller, M. (1954). *J. gen. Microbiol.* **11**, 57–58.

A Ninth Variety of *Paramecium aurelia*

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SUMMARY: A new variety of *Paramecium aurelia*—variety 9—containing two mating types XVII and XVIII, has been identified from seven samples of water collected in Scotland. Variety 9 belongs to the group A varieties in the Sonneborn system of classification, but shows no intervarietal mating reactions.

Paramecium aurelia is readily distinguished from other species of *Paramecium* by morphological characters, such as the structure and number of micronuclei (see Wenrich, 1928). Within the species *P. aurelia*, however, no fewer than eight distinct varieties or physiological subspecies have been recognized (Sonneborn, 1938, 1942; Sonneborn & Dippell, 1946*a*, *b*). The organisms are classified into one or other of these varieties according to mating behaviour: within a variety, conjugation may be readily brought about between the two mating types which occur in all varieties (except no. 7), and leads to the production of viable progeny; between varieties, by contrast, conjugation may not occur at all, or if it does, the F_1 hybrids, or the F_2 progeny derived from them, are non-viable.

Recently we have collected from a number of localities in Scotland samples of paramecia which have been identified as *Paramecium aurelia* from the two small vesicular micronuclei seen to be present in stained preparations. The organisms were collected as follows: stock no. 503, St Margaret's Loch, Edinburgh; no. 504, Figgate's Pond, Edinburgh; no. 505, Strathblane, near Glasgow (all November 1952); no. 506, canal at Kingsknowe, Edinburgh; no. 507, canal at Colinton, Edinburgh (February 1953); no. 509, canal at Ratho Park, Edinburgh (March 1953); no. 510, Blackford Pond, Edinburgh (October 1953). Cultures were established by multiplication of a single organism from each stock collected, and in course of time conjugation was seen to occur within such cultures. Tests showed that the same two mating-types were present in all these stocks, but none of the organisms of these two mating types could be induced to show the agglutinative mating reaction, even weakly, with organisms of any of the eight known varieties. Hence, the stocks listed in Table 1 were placed in a new variety of *P. aurelia*—variety 9, which forms the two mating types XVII and XVIII.

Analysis of ex-conjugants showed that the inheritance of mating-type is of the kind named by Sonneborn (see 1947) *caryonidal*, that is, from each pair of conjugating organisms, four caryonides are formed by a single division of the ex-conjugants, and the mating-types of all organisms within a caryonide are the same. Different caryonides may, however, be of different mating-types. By its manifestation of caryonidal inheritance of mating-type, variety 9 is classified amongst the group A varieties (nos. 1, 3, 5 and 7) of *Paramecium aurelia*.

From one group of conjugants which had been kept at 25°, twenty-five caryonides were obtained of one mating-type, and fifteen of the other mating-type, which accords well enough with Sonneborn's formula of $18{\cdot}4 + t/0{\cdot}51$ for the percentage of mating-types of even number in varieties 1 and 3 (t being the temperature in degrees Centigrade; Sonneborn, 1947). In view of the absence of any cross-reactions with mating types of other varieties, we arbitrarily denote that mating type which is formed in excess of 50% after conjugation at 25° (or higher) as type XVIII, and the other one as type XVII.

Mating of variety 9 organisms will occur when growth has previously been at temperatures between 18 and 25°, though not at higher temperatures, and conjugation has been observed to occur at various times of the day, and after growth in continuous darkness. Lastly, it may be added that the inter-autogamous period is about 12 days, similar to that found in variety 1.

Variety 9 is the only variety of *Paramecium aurelia* not yet found in North America.

We wish to thank Dr R. V. Dippell for cytological help in this work.

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The Distribution of Polysaccharide Production in Aerobacter and Escherichia Strains and its relation to Antigenic Character

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With a note on the Influence of Potassium Deficiency upon Production of Polysaccharide by *Aerobacter aerogenes*

By J. P. Duguid and J. F. Wilkinson

SUMMARY: Strains of *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli*, including mucoid capsulate, mucoid non-capsulate, smooth and rough variant forms, were grown on defined agar media. The polysaccharide content of the cultures was estimated by the anthrone method and related to the amount of growth as measured by the non-dialysable nitrogen content. Polysaccharide production, as measured by the polysaccharide:nitrogen ratio, was increased in all strains when growth was limited by a restricted supply of nitrogen source so that added carbohydrate remained in relative excess. A low incubation temperature, 15–20° instead of 35°, influenced polysaccharide production differently in the various strains and species; thus it greatly increased production in *E. coli* strains, but decreased it in *A. cloacae* strains grown on certain media. The various strains showed considerable differences in the amount of polysaccharide production and in the distribution of the polysaccharide between the different possible sites of accumulation: i.e. intracellular, capsular and as loose slime. The mucoid strains produced the largest amounts of polysaccharide, most of which was present extracellularly as capsules or slime; the smooth strains produced much smaller amounts, almost entirely intracellular; a rough strain produced least of all. The antigenic constitution of certain strains was examined, particularly that of a mucoid capsulate *A. aerogenes* and mucoid non-capsulate and non-mucoid smooth variants derived from it. The designation of such morphological and immunological variants is discussed.

The amount of polysaccharide produced varies greatly with different strains and species of Enterobacteriaceae. Among twelve strains of *Escherichia coli* grown at 13–17° on glucose medium, Morgan & Beckwith (1939) found that the amount of polysaccharide produced, as indicated by the degree of mucoidness, was large in five strains, moderate in two strains and small in the remainder; they found similar differences among salmonella strains. Mucoid, smooth and rough variants of the same strain may also show great differences in polysaccharide production. Boivin, Mesrobeanu, Magheru & Magheru (1936) measured the amount of a polysaccharide-containing substance, the 'complete antigen', which was extracted with trichloroacetic acid from cultures of *E. coli*. Large amounts of this (0·41–0·98 mg./100 mg. moist culture) were found in all of six 'smooth' strains which gave smooth colonies and were not agglutinable by trypaflavine, while none was found in five of six 'rough' strains which gave irregular granular colonies and were trypaflavine-agglutinable. Certain trypaflavine-agglutinable strains of intermediate colony type contained considerable amounts of the substance. Lankford, Hoyo & Lutteringer (1951), using the periodate-Schiff staining method, found that O-inagglutinable rough variants of salmonella and shigella strains lacked the stainable intracellular polysaccharide of the smooth parent strains; they therefore suggested that this polysaccharide corresponded to the hapten of the O antigen characteristic of the smooth form.

Duguid & Wilkinson (1953) studied the influence of different cultural conditions on the amount of polysaccharide produced by a single strain (A3) of *Aerobacter aerogenes*, distinguishing the polysaccharide situated intracellularly, in the capsules or in the loose extracellular slime. In the present investigation the same methods have been applied for examination of several different strains of *A. aerogenes*, *A. cloacae* and *Escherichia coli.* For each strain the effect was observed of variation in the sugar:nitrogen ratio of the medium and of variation in the temperature of incubation.

METHODS

Strains. The strains labelled 'A' were isolated in this laboratory from faeces or water. Those designated NCTC were obtained from the National Collection of Type Cultures, Colindale, London. The strains were identified by fermentation and other biochemical tests as described by Wilson (1935). Also, cultures on MacConkey's lactose peptone agar at 35° and at 15–20° were observed for colony form (mucoid, smooth or rough), and were examined by the wet-film indian ink method for the presence of capsules and loose slime (Duguid, 1951). The organisms were suspended in an aqueous solution of acriflavine (0·2 %, w/v) and sodium chloride (0·85 %, w/v) and were observed during several hours for the occurrence of spontaneous agglutination, indicative of the rough form.

The strains A1 and A3 of *Aerobacter aerogenes* corresponded with Wilson's *Bacterium aerogenes* type I. They had the following properties: capsulate and slime-forming, mucoid (Pl. 1, fig. 1), non-motile, methyl-red negative, Voges-Proskauer positive, utilized citrate, indole negative, Eijkman negative, did not liquefy gelatin, fermented glucose, lactose, sucrose, mannitol, inositol and glycerol, producing acid and gas. Two stable variants were derived from strain A3 which resembled the latter exactly in their biochemical reactions, but differed in their mode of polysaccharide production. Variant strain A3 (Sl) originated on several occasions in growths of strain A3 in a defined liquid medium. It differed from A3 in being non-capsulate; it was slime-forming and gave mucoid growths which were unusually tough and sticky. Variant strain A3 (O) was obtained from strain A3 cultivated in broth containing homologous antiserum; it differed from A3 in being non-capsulate and non-slime-forming, and in giving smooth non-mucoid growths (Pl. 1, fig. 2). Strain D301 (R) (kindly supplied by Prof. E. M. Dunlop) was a non-capsulate and non-slime-forming rough colony variant (Pl. 1, fig. 3) of an *A. aerogenes* isolated from drinking water; it resembled strain A3 in most biochemical reactions and in being non-motile, but differed in fermenting inositol and glycerol without gas production and in being agglutinable by acriflavine. Strain NCTC 5920 was a typical *A. cloacae* in being motile, mucoid, slime-forming and mainly non-capsulate; it resembled *A. aerogenes* (A3) in biochemical reactions, except that it liquefied gelatin and failed to produce gas in fermenting inositol and glycerol. Strain NCTC 5936 was supplied as '*Bacterium aerogenes* type I'; although not liquefying gelatin, it is treated here as *A. cloacae* which it resembled in being motile, slime-forming and non-capsulate, in failing to produce gas on fermentation of inositol and glycerol, and in its antigenic character.

The *Escherichia coli* strains corresponded to Wilson's *Bacterium coli* type I. They had the following properties: methyl-red positive, Voges-Proskauer negative, did not utilize citrate, indole positive, Eijkman positive, did not liquefy gelatin, fermented glucose, lactose, mannitol and glycerol producing acid and gas, but not inositol. Strains A55 and A102 were motile, and A49, A93 and A108 non-motile. Strain A93 was capsulate and mucoid, strains A49 and A102 were non-capsulate and, at 15–20°, slime-forming and mucoid, and strains A55 and A108 were non-capsulate and non-slime-forming, giving smooth non-mucoid colonies. Strain A49 (O) was a non-slime-forming, smooth colony variant derived from A49. Strain A108 (R) was a rough colony variant derived from A108; it was not agglutinable by acriflavine and was classed as a smooth-rough intermediate.

Culture methods. The strains were grown as described by Duguid & Wilkinson (1953) on cellophan disks over agar medium in open Petri dishes which were incubated in sealed tanks to ensure adequate aeration and constant humidity. Each strain was grown on a series of plates of 50 ml. medium containing different amounts of ammonium sulphate as the sole nitrogen source; each 50 ml. medium also contained: 250 mg. lactose; 500 mg. phosphate (Na_2HPO_4, 3 parts by weight, KH_2PO_4, 1 part; pH = 7·3); 100 mg. NaCl; 50 mg. K_2SO_4; 10 mg. $MgSO_4.7H_2O$; 1 mg. $CaCl_2.6H_2O$; 0·05 mg. $FeSO_4$; 1·0 g. agar powder (one batch of Japanese agar obtained pre-1939 was used for all media). Incubation was continued for 4 days at 35° or 14 days at 15–20°, these durations being found sufficient for completion of growth and full polysaccharide production.

Estimations. Measurements were made as described by Duguid & Wilkinson (1953) of the final pH value, the degree of mucoidness and the wet weight of the culture; the total cell count and average capsule diameter after suspension in saline solution; the dry weight, total nitrogen content and polysaccharide content after dialysis of the saline suspension. Polysaccharide estimations by the anthrone method were made separately for centrifuged deposits and supernatants; the deposits contained the intracellular and capsular polysaccharides together, and the supernatants the loose slime polysaccharide. A separate estimate of the intracellular polysaccharide (that in the cell but not capsule) was made according to the intensity of staining by the periodate-Schiff method of Hotchkiss (1948). The amount of polysaccharide production relative to the amount of bacterial protoplasm was estimated as the ratio of the polysaccharide content of the culture to the non-dialysable nitrogen content (polysaccharide:nitrogen ratio). The degree of mucoidness was observed visually and was also measured as the ratio of the wet weight of the culture to its nitrogen content (wet weight:nitrogen ratio). The average length and width of the bacilli, not including the capsule, were calculated from measurements of 50–100 cells in a phase-contrast photomicrograph of an unstained wet film. The *total cell and capsule volume* of the culture suspended in saline solution was calculated as $(\frac{4}{3}\pi R^3 + \pi R^2(L-1)) \times$ total cell count, and the *total cell volume* (excluding capsules) as $\pi r^2 L \times$ total cell count, where R = half the 'average capsule diameter', r = average bacillus radius and L = average bacillus length (measurements in microns). For comparison, the *volume of the surface culture before suspension* was calculated as the wet weight converted from mg. to mm.^{3} (assuming specific gravity = 1·1). This volume included the slime as well as the cells and capsules. In the surface culture the capsules are less hydrated and occupy a smaller volume than after suspension in saline solution (Duguid & Wilkinson, 1953).

Serological methods. Cultures were grown for 24 hr. at 35° on agar containing 0·5 g. lactose and 0·5 g. peptone/100 ml. Whole-culture suspensions (cells, capsules and slime) were made in saline solution to a density of about 10^9 cells/ml. Rabbits were injected with such suspensions to prepare specific antisera for strains A3 and A3 (O). The antigens tested *in vitro* were, for strain A3 (O), the whole-culture suspension and, for strains A3 and A3 (Sl), the separated cellular and slime fractions. The latter were obtained by high-speed centrifugation of the whole-culture suspension. The supernatant first separated was taken as a cell-free preparation of slime. The cellular deposit was washed by suspension in fresh saline and re-centrifugation, this being done 3 times for strain A3 and 6 times for the more viscous strain A3 (Sl). Finally, a very dense suspension of the deposit was made in saline containing 0·08 g. sodium azide/100 ml. The A3 deposit provided 'washed capsulate cells' and the A3 (Sl) deposit 'washed non-capsulate cells', in both cases slime-free.

For agglutination tests the whole-culture suspension or the washed cellular deposit was diluted to about 3×10^8 cells/ml. and mixed with equal volumes of antiserum in a range of doubling dilutions. The results were observed after incubation for 2 hr. at 37°, and again after standing overnight at 4°. For a capsulate strain such as A3, agglutination occurred only with low dilutions of antiserum and gave rise to compact floccules. For a non-capsulate strain such as A3 (O), agglutination usually occurred to a high titre and gave fine granules. 'Capsule swelling' tests were made by mixing a drop of diluted antiserum with the cell suspension and examining a wet film of the mixture microscopically. Precipitation tests were made to demonstrate the reaction of antiserum with the loose slime polysaccharide. Doubling dilutions of the antiserum in saline, from 1/2 to 1/32, were placed in narrow tubes and a 1/10 dilution of the culture supernatant was superimposed. The tubes were examined after 15 min. at 37°, when a positive reaction was indicated by the appearance of a white ring of precipitate at the interface.

For agglutinin-absorption and precipitin-absorption tests the absorbing reagent was added drop by drop to an equal volume of the antiserum, with constant agitation, and the mixture was incubated for 30 min. at 37°. After removal of the precipitated cells or slime by centrifugation, the supernatant was tested with the appropriate antigen to show whether the 'capsule swelling', agglutinating or precipitating activity had been lost as a result of the absorption. In the 'capsule swelling' tests the minimum dilution tested was that of the absorbed serum (i.e. 1/2), while in the agglutination tests it was 1/10 for the A3 cells and 1/40 for A3 (Sl) and A3 (O) cells.

RESULTS

Polysaccharide production was observed in the various strains of *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* grown on 50 ml. plates of medium containing 250 mg. lactose and different amounts of ammonium sulphate, from 2·5 to 250 mg. Representative results for the media with 75 and 7·5 mg. ammonium sulphate/50 ml. are shown in Table 1. For a given supply of ammonium sulphate all the strains gave nearly the same amount of growth as measured by the nitrogen content. In all cases the cultures with 75 mg. ammonium sulphate/50 ml. gave growth which was limited by exhaustion of the lactose and gave nearly minimal production of polysaccharide. This was shown by the finding that with 250 mg. ammonium sulphate/50 ml. the amount of growth was not greater, nor the polysaccharide: nitrogen ratio less. The cultures with 7·5 mg. ammonium sulphate/50 ml. gave growth which was limited by exhaustion of this sole nitrogen source, and gave maximal or nearly maximal production of polysaccharide. This was shown by the finding that cultures on 2·5 mg. ammonium sulphate/50 ml. gave a third the amount of growth, but hardly any further increase in the polysaccharide: nitrogen ratio in spite of their threefold greater excess of lactose. All the strains of *A. aerogenes*, *A. cloacae* and *E. coli* thus showed an upper limit to polysaccharide production in the same way as strain A3 (Duguid & Wilkinson, 1953); increase of the sugar: nitrogen ratio in the medium gave an increase of polysaccharide production only up to a maximal level and any further excess of sugar remained unconsumed.

While all strains showed this tendency to give increased polysaccharide production on media with a restricted supply of ammonium sulphate, they differed considerably in the amount of polysaccharide formed and in its distribution between the different possible sites of accumulation. Mucoid cultures were given by all the strains which formed capsules or loose slime in considerable amounts. The degree of mucoidness as observed visually and as measured by the wet weight: nitrogen ratio was increased in proportion to the amount of capsular and slime polysaccharide, but not according to the amount of intracellular polysaccharide. The degree of mucoidness corresponded to the extent whereby the volume of the surface culture (including capsules and slime) exceeded the total volume of the cells (not including capsules and slime). In the non-mucoid cultures of strains A3 (O), D301 (R), A55 and A102, the calculated total cell volume was found to be approximately equal to the surface culture volume as measured by wet weight. In the slightly mucoid cultures of strains A1, A3, A3 (Sl), NCTC 5920, NCTC 5936, A93 and A102 grown on the ammonia-rich media, the cells occupied from 13 to 44 % of the surface culture volume. In the highly mucoid cultures of these strains grown on the ammonia-poor media, the cells occupied only 1–5 % of the surface culture volume. Similarly, with increasing mucoidness the dry weight comprised a decreasing proportion of the wet weight of the culture; this proportion was 22–37 % in the non-mucoid cultures, 10–14 % in the slightly mucoid cultures and 3–6 % in the highly mucoid cultures.

Capsulate Aerobacter aerogenes *strains A* 1 *and A* 3

Strain A3 gave results which were typical of the majority of *A. aerogenes* strains. Strain A1 differed from it in forming much larger capsules and in giving colonies of a tougher and stickier consistency. Comparison of the results given in Table 1 shows that the amount of growth and the total amount of polysaccharide production were similar in the two strains. However, strain A1 formed less polysaccharide intracellularly and more as loose slime. The average capsule diameter as measured after suspension in saline was twice as great in strain A1 as in strain A3. Correspondingly, the calculated total volume of cells and capsules in suspension was about eightfold greater for A1 than for A3. The larger capsular volume of strain A1 was not due to a greater production of capsular polysaccharide, but to a greater hydration and swelling of this material on suspension in water or saline solution. The great extent of this capsular expansion is shown by comparison of the volume of the surface culture before suspension (i.e. wet weight mg. converted to mm.^{3}) with the calculated total volume of cells and capsules after suspension. The latter volume, although not including the loose slime, exceeded the former volume very greatly (about eightfold) in strain A1, but only slightly in strain A3 (Table 1). In spite of the great difference in physical character, the capsular polysaccharides of strains A1 and A3 were found to be identical antigenically; capsule-swelling (Pl. 1, fig. 4) and agglutination tests showed that both strains belonged to the klebsiella capsule type 54 of Edwards & Fife (1952), and cross-absorption tests failed to reveal any antigenic difference. Moreover, the O antigens of strains A1 and A3 were found to be identical by agglutinin-absorption tests on non-mucoid smooth variants of these strains. As reported previously (Duguid & Wilkinson, 1953), lowering of the incubation temperature from 35° to 15–20° gave little increase of polysaccharide production by strain A3: production was barely doubled in the ammonia-rich cultures and not increased at all in the ammonia-poor.

Aerobacter aerogenes *strain A* 3, *its slime-forming variant A* 3 (*Sl*), *its 'smooth' variant A* 3 (*O*) *and the 'rough' strain D* 301 (*R*)

The antigenic composition of strain A3 and its variants A3 (Sl) and A3 (O) was studied serologically. The results shown in Table 2 indicate the following facts. (1) The capsular and slime polysaccharides of strain A3 are antigenically similar or identical with each other and also with the slime polysaccharide of strain A3 (Sl). (2) The cells of strains A3, A3 (Sl) and A3 (O) all possess the same somatic ('O') antigen; this lies uncovered at the

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Table 1. *Polysaccharide production by aerogenes, cloacae and coli strains grown on 50 ml. amounts of medium containing 250 mg. lactose, 500 mg. phosphate at pH 7·3 and different amounts of ammonium sulphate*

Species	Strain	Ammonium sulphate in medium (mg.)	Degree of mucoid-ness	Capsule diameter (μ)	Int. poly. stain*	Cell count $\times 10^{-9}$	Calculated total volume (mm.^{3})†		Wet weight (mg.)	Total nitrogen (mg.)	Polysaccharide (mg.)		Wet weight (mg./mg. nitrogen)	Poly-saccharide (mg./mg. nitrogen)
							Cells only	Cells and capsules			Deposit	Super-natant		
						(*a*) Incubated for 4 days at 35°								
Aerogenes	A 1	75	⊥	3·3	⊥	430	155	8,600	1,170	8·3	10·8	7·9	140	2·3
		7·5	+++	9·8	+	40	32	21,300	2,350	1·6	16·1	24·6	1,500	26·0
	A 3	75	⊥	1·6	⊥	520	270	1,580	930	7·4	10·6	2·7	130	1·8
		7·5	+++	4·3	++	54	45	2,630	2,410	1·5	22·5	20·8	1,600	29·0
	A 3 (Sl)	75	⊥	–	⊥	550	226	226	1,040	8·6	3·8	9·7	120	1·5
		7·5	+++	–	++	44	37	37	3,300	1·5	3·6	45·0	2,200	32·0
	A 3 (O)	75	–	–	⊥	800	320	320	340	9·6	5·3	0·7	35	0·63
		7·5	–	–	+++	68	59	59	60	1·5	3·8	0·3	41	2·8
	D 301 (R)	75	–	–	–	1000	300	300	340	9·9	1·8	0·3	34	0·21
		7·5	–	–	⊥	94	57	57	70	1·4	0·25	0·34	49	0·41
Cloacae	NCTC 5936	75	⊥	–	⊥	510	133	133	780	7·1	5·0	8·4	110	1·9
		7·5	++	–	+	64	37	37	750	1·5	1·9	5·7	500	5·1
	NCTC 5920	75	–	–	⊥	610	141	141	320	9·2	5·3	1·0	35	0·69
		7·5	++	–	+	130	39	39	1,160	1·6	1·2	16·3	730	11·0
Coli	A 93	75	⊥	2·5	⊥	460	161	3,960	1,270	9·3	12·3	1·8	140	1·5
		7·5	++	3·3	+	53	33	1,190	1,230	1·5	6·9	5·4	810	8·1
	A 102	75	–	–	⊥	620	286	286	440	9·2	5·4	0·4	48	0·64
		7·5	–	–	+	76	44	44	70	1·4	1·8	0·3	49	1·5
	A 55	75	–	–	⊥	800	408	408	450	9·3	6·2	0·8	48	0·75
		7·5	–	–	++	79	46	46	50	1·4	1·2	0·1	35	0·90
						(*b*) Incubated for 14 days at 15–20°								
Coli	A 102	75	⊥	–	⊥	720	302	302	1,060	9·1	5·7	4·9	120	1·2
		7·5	++	–	+	55	32	32	990	1·6	1·7	8·4	640	6·5
	A 55	75	–	–	+	690	357	357	540	8·8	13·1	0·6	61	1·6
		7·5	–	–	++++	76	51	51	90	1·5	5·5	0·3	61	4·0
	A 49	7·5	++	–	++	68	.	.	970	1·5	1·4	8·7	640	6·7
	A 49 (O)	7·5	–	–	++	53	.	.	160	1·5	2·4	1·4	110	2·6
	A 108	7·5	–	–	++	49	.	.	200	1·5	2·3	1·4	130	2·4
	A 108 (R)	7·5	–	–	++	32	.	.	50	1·4	2·2	0·3	35	1·8

* Intracellular polysaccharide staining intensity, –, ⊥, + to ++++.

† I.e. when in suspension in saline solution.

Note. In all cultures the final pH was in range 6·5–7·0, and mean bacillus length in range 1·3–1·9 μ.

surface of A3 (O) and A3 (Sl) cells, but is entirely occluded by the capsule in A3 cells. (3) The A3 (O) whole cultures and the washed A3 (Sl) cells are devoid of any A3 capsular or slime antigen.

Table 2. *Highest dilutions of antisera giving precipitation, agglutination and capsule-swelling reactions with various antigen preparations of* Aerobacter aerogenes

		A3 antiserum absorbed with						A3 (O) antiserum absorbed with	
Antigen	Test*	Nil	A3 slime	A3 washed capsulate cells	A3 (Sl) slime	A3 (Sl) washed cells†	A3 (O) cells†	Nil	A3 (Sl) washed cells
A3 slime	P	16	0	0	0	8	16	0	0
A3 capsulate cells	A	20	0	0	0	10	20	0	0
	CS	8	0	0	0	8	8	0	0
A3 (Sl) slime	P	16	0	0	0	8	16	0	0
A3 (Sl) washed cells†	A	640	640	640	640	0	0	20,480	320
A3 (O) cells† (whole culture)	A	640	640	640	640	0	0	10,240	160

* P = precipitation; A = agglutination; CS = capsule-swelling. † I.e. non-capsulate.
Capsule-swelling titres are initial dilutions before mixing with antigen; agglutination titres are final dilutions.

Aerobacter aerogenes *strains A3 and A3 (Sl)*

As compared with the parent strain A3, strain A3 (Sl) differed in being non-capsulate and in giving colonies which were much tougher and stickier in consistency, though of similar mucoid appearance. While the total amount of polysaccharide produced was almost the same in the two strains, its distribution was quite different. Strain A3 (Sl) gave over twice as much polysaccharide in the loose slime fraction and correspondingly less in the cellular deposit; presumably this was the consequence of its failure to retain extracellular polysaccharide as capsules. The amount of intracellular polysaccharide revealed by periodate-Schiff staining was about the same in the two strains. It is thus assumed that the intracellular polysaccharide content of the A3 cultures was equal to the whole of the deposit polysaccharide content of the A3 (Sl) cultures. On this basis it is estimated that in strain A3 the proportion of the deposit polysaccharide occurring in the capsules was about 65 % in the ammonia-rich cultures and 85 % in the ammonia-poor, the remainder occurring intracellularly. Although A3 (Sl) slime was identical antigenically with A3 slime, it differed physically in being much more viscous in solutions of equal concentration and in failing to increase the surface charge on red cells so as to inhibit viral haemagglutination (Macpherson, Wilkinson & Swain, 1953).

Aerobacter aerogenes *strain A3 (O)*

As compared with the parent strain A3, strain A3 (O) differed in being non-mucoid, smooth in colony form, non-capsulate and non-slime-forming. It gave a similar or slightly greater amount of growth than A3, but a much smaller total production of polysaccharide. Most of its polysaccharide (about 90 %) was found in the deposit fraction, and, since capsules were absent, all of this must have been situated intracellularly. Indeed, periodate-Schiff staining showed that the intracellular polysaccharide content of strain A3 (O) was slightly greater than that of strain A3. If, as suggested by Lankford *et al.* (1951), this 'intracellular' polysaccharide corresponds to the 'O' somatic antigen, some of it must permeate the cell wall and lie exposed on the surface. The small amount of free extracellular polysaccharide found in the supernatant fraction of A3 (O) cultures may have been some liberated 'O' polysaccharide; at least it did not correspond to the capsular and slime polysaccharide antigens of strain A3, since serological tests showed that A3 (O) cultures were devoid of these. The polysaccharide: nitrogen ratio of strain A3 (O) was increased about fivefold in ammonia-poor cultures as compared with ammonia-rich (compare 15- to 20-fold increases for A3 and A3 (Sl)). This increase was not accompanied by any increase in the degree of mucoidness, presumably because it is only extracellular polysaccharide which confers the mucoid character. That A3 (O) cultures did not contain any appreciable volume of extracellular material was confirmed by the close approximation of the calculated total cell volume with the surface culture volume (wet weight).

Aerobacter aerogenes *strain D301 (R)*

The rough *Aerobacter aerogenes* strain D301 (R) was also non-capsulate and non-slime-forming. It differed from strain A3 (O), not only in the rough character of its colonies, but also in its much smaller production of intracellular polysaccharide as shown by the virtual absence of staining by the periodate-Schiff method. This rough strain produced less polysaccharide than any smooth or mucoid strain, and it exhibited a minimal, barely twofold, increase of the polysaccharide: nitrogen ratio on restriction of the ammonium sulphate supply.

Aerobacter cloacae *strains NCTC 5920 and 5936*

The two strains of *Aerobacter cloacae* gave generally similar results. They formed slime and intracellular polysaccharide, but not capsules. (In NCTC 5920 cultures at 15–20° a proportion of the cells showed soft, indefinitely demarcated capsules which slowly disintegrated and dispersed in wet indian ink films (see Pl. 1, fig. 5)). Limitation of the ammonium sulphate supply increased the amount of polysaccharide production in both strains, though to a lesser extent in NCTC 5936. The maximal production was not as great as in the mucoid *A. aerogenes* strains. As compared with the 35° cultures (Table 1), those at 15–20° gave similar amounts of growth, but differed in the amount of polysaccharide production. At the lower temperature the polysaccharide: nitrogen ratio of NCTC 5936 cultures, ammonia-poor and ammonia-rich, was decreased to about a third; that of NCTC 5920 cultures was decreased only slightly if ammonia-poor, and increased about threefold if ammonia-rich. When tested with an antiserum prepared for the typical gelatin-liquefying *A. cloacae* strain U8 (described by Edmunds, 1954) the slimes of strains NCTC 5920 and 5936 both gave precipitation to the significant titre (1/16). In precipitin absorption tests, the U8 antibodies were absorbed entirely by NCTC 5936 slime, though not entirely by NCTC 5920 slime.

Escherichia coli *strains*

Tests were made with representative strains of the three varieties of *Escherichia coli* distinguished by Duguid (1951), the rare mucoid capsulate variety (A 93), the fairly common mucoid slime-forming non-capsulate variety (A 49 and A 102), and the very common non-mucoid variety, which is non-capsulate and not markedly slime-forming (A 49 (O), A 55 and A 108). The capsulate *E. coli* strain A 93 gave results similar to those of the capsulate *Aerobacter aerogenes* strain A 3, forming capsules and slime almost as abundantly at 35° as at 15–20°. The increase of polysaccharide:nitrogen ratio obtained on limitation of the ammonium sulphate supply was, however, only a third as great as in strain A 3.

The *Escherichia coli* strains A 49 and A 102, when grown at 15–20°, gave mucoid growths with abundant slime formation; their cultures then resembled those of the slime-forming non-capsulate *Aerobacter cloacae* strains. When grown at 35°, however, these *E. coli* strains produced very much less slime polysaccharide, so that their cultures appeared quite non-mucoid. With strain A 102 the lowering of incubation temperature from 35° to 15–20° increased the total polysaccharide production by twofold for ammonia-rich cultures and fourfold for ammonia-poor. Similar large increases were obtained with several other slime-forming *E. coli* strains. These strains resembled the strains found by Morgan & Beckwith (1939) and Beiser & Davis (1953) to give mucoid growths only at low temperatures. The small amount of slime polysaccharide formed in the non-mucoid growths at 35° appeared to be identical antigenically with that formed in large amounts in the mucoid growths at 15–20°. A rabbit was immunized by injection of a non-mucoid A 102 culture grown at 35° on the ammonia-rich medium; it showed abundant production of precipitating antibodies for the slime of a mucoid A 102 culture grown at 15–20° on the ammonia-poor medium.

The *Escherichia coli* strains A 49 (O), A 55 and A 108 gave results which were generally similar to those of the non-mucoid *Aerobacter aerogenes* strain A 3 (O). While incubation at 15–20° did not induce formation of slime in appreciable amount, it increased the production of intracellular polysaccharide. Decrease of the incubation temperature and limitation of the ammonium sulphate supply increased the total polysaccharide production almost as much in strain A 55 as in strain A 102. In A 55, however, the main increase occurred in the intracellular (deposit) fraction, and in A 102 in the slime (supernatant) fraction. The small intracellular polysaccharide content of A 55 grown at 35° on ammonia-rich medium is shown in Pl. 1, fig. 6, the moderate content of the growth at 35° on ammonia-poor medium in Pl. 1, fig. 7, and the large content of the growth at 15–20° on ammonia-poor medium on Pl. 1, fig. 8. As compared with its slime-forming parent strain A 49, non-slime-forming variant A 49 (O) showed an increase of intracellular polysaccharide. Loss of the power to form polysaccharide extracellularly led to increased storage of polysaccharide intracellularly.

The *Escherichia coli* smooth strain A 108 and its rough variant A 108 (R) did not differ in the same manner as the *Aerobacter aerogenes* smooth strain A 3 (O) and the rough strain D 301 (R). Strain A 108 (R) differed from A 108 only in forming less slime polysaccharide. Unlike D301 (R) it formed a considerable amount of intracellular polysaccharide and was not agglutinable by acriflavine. It thus appeared that A108 (R) was a smooth-rough intermediate and not a true rough strain as D301 (R).

DISCUSSION

The present findings show that polysaccharide production is greatly increased under conditions of nitrogen limitation and carbohydrate excess, not only with mucoid capsulate *Aerobacter aerogenes* strains as found by Duguid (1948) and Duguid & Wilkinson (1953), but also in mucoid non-capsulate, non-mucoid smooth and non-mucoid rough strains of *A. aerogenes*, and in strains of *A. cloacae* and *Escherichia coli*. However, these latter strains differed markedly in the amount of polysaccharide produced and in the proportions accumulated as capsules, as loose slime and intracellularly. For each strain there were lower and upper limits to polysaccharide production: a minimum which was still produced when growth was limited by exhaustion of carbohydrate in the medium and a maximum which was not exceeded even when unconsumed carbohydrate remained available after cessation of growth due to nitrogen starvation. The difference between the maximal and minimal productions (polysaccharide:nitrogen ratios) was greater in the mucoid strains (e.g. 5–20-fold) than in the non-mucoid smooth and rough strains (e.g. 2–4-fold).

The mucoid strains produced the largest amounts of polysaccharide, forming much of this extracellularly as capsules or slime (50–90 %); the non-mucoid smooth strains produced smaller amounts, mostly intracellularly; the one completely rough strain produced the smallest amount, lacking even an appreciable intracellular content. The amount of polysaccharide which could be formed extracellularly was much greater than that which could be formed intracellularly. For the *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* strains the maximal intracellular polysaccharide yields were respectively 2·5, 1·3 and 3·7 mg./mg. culture nitrogen, while the maximal extracellular polysaccharide yields were 30, 10 and 6 mg./mg. nitrogen.

Colony form, whether mucoid (M), smooth (S) or rough (R), may serve as an indication of the classes of antigens, capsular (K), somatic 'smooth' (O) and 'rough' (R), which the bacterium is genetically conditioned to produce. However, the relationship is not invariable since colony form is partly determined by cultural conditions. Confusion can thus arise when bacterial variants differing in antigen production are named according to colony form. The difficulties in the case of pneumococci have recently been emphasized by Austrian (1953). He points out that capsulate strains which produce the K polysaccharide in large amounts give mucoid colonies, while those producing K polysaccharide in small amounts give smooth colonies indistinguishable in appearance from the colonies of non-capsulate K-free strains. Accordingly, capsulate and non-capsulate pneumococci have been classified respectively as 'smooth' and 'rough' by some authors and as 'mucoid' and 'smooth' by others. The same difficulty is encountered in *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* strains. In the experiments shown in Table 1, the capsulate and slime-forming strains yielded mucoid growths on the ammonia-poor media which gave a large amount of polysaccharide, but non-mucoid smooth growths on the ammonia-rich media which gave a small amount. In the case of *E. coli* strain A102, serological tests confirmed that the non-mucoid culture grown at 35° contained small amounts of the same antigen (K) as was present abundantly in the slime of the mucoid culture grown at 15–20°.

The value of colony appearance as an indicator of antigen production is thus limited. If a culture is obviously mucoid under favourable cultural conditions it may be assumed to form K antigen, but if it is non-mucoid it may or may not form K antigen and this must be decided by serological tests. As suggested by Kauffmann (1949) the antigenic variants should be designated according to the classes of antigens which they produce rather than according to their colony form. In addition to the capsular (K) antigens, Kauffmann proposed a second class of extracellular antigens, the 'mucoid' (M) antigens which are non-capsular and occur as loose slime not firmly occluding the O antigen. He thus distinguished eight variant forms: MKO, KO, MO, O, MKR, KR, MR and R. The loose slime polysaccharides observed in the present series of strains apparently correspond to Kauffmann's M antigens. The slime of strain A3 (Sl), for instance, was shown to differ from the K antigens in failing to render the unheated bacilli inagglutinable by O antibody (A3 (O) antiserum). We suggest, however, that the M antigens should not be distinguished in a major class apart from the K antigens which they resemble very closely. As defined by Kauffmann (1947), the K class already includes morphologically different varieties of antigen, L, A and B; the L and B antigens, although occluding the O antigens, are not associated with demonstrable capsules like the A antigens. Pickett & Cabelli (1953) also described a further K antigen called Sm, obtained after prolonged immunization, which is common to all mucoid forms of Friedländer's bacillus, being present in addition to their main type-specific K antigen. The K class might conveniently be widened further to include the slime antigens and haptens. Although differing morphologically, the slime antigens resemble the capsular antigens chemically and serologically. For instance, the similarity in antigenic specificity of slime and capsules was demonstrated for a klebsiella strain by Edwards & Fife (1952) and for strains A3 and A3 (Sl) in the present investigation. We therefore suggest that the antigenic variants be classified immunologically in four groups only: 'K' forms which produce capsular or slime antigens (e.g. type-specific M, L, A or B antigens) and are distinguished as 'KO' or 'KR' according to whether or not the group-specific O antigen is also produced; 'O' forms producing O antigen but not K antigen; 'R' forms not producing either O or K antigens. Thus strains A1, A3 and A3 (Sl) are KO forms, strain A3 (O) is an O form, and D.301 (R) is an R form.

The morphological classification of variants should be stated quite separately, defining the colony form as mucoid, smooth or rough, and the cell form as capsulate and slime-forming, non-capsulate and slime-forming or non-capsulate and non-slime-forming. The cultures for these observations should be made on media with a high carbohydrate:nitrogen ratio, both at 35° and at 15–20° (Duguid, 1951; Duguid & Wilkinson, 1953).

Note on the Influence of Potassium Deficiency upon Production of Polysaccharide by *Aerobacter aerogenes*

By J. P. Duguid and J. F. Wilkinson

In a previous investigation (Duguid & Wilkinson, 1953) it was found that polysaccharide production by *Aerobacter aerogenes* strain A3 was greatly increased when growth in a lactose-containing medium was limited by deficiency of the nitrogen, phosphorus or sulphur sources. Observations have now been made on cultures where growth was limited by deficiency of potassium. The cultures were grown aerobically for 4 days at 35° on 50 ml. agar medium containing 250 mg. lactose, 250 mg. ammonium sulphate, 500 mg. phosphate (Na_2HPO_4, 3 parts by weight; NaH_2PO_4, 1 part), 100 mg. NaCl, 10 mg. $MgSO_4.7H_2O$, 1 mg. $CaCl_2.6H_2O$, 0·05 mg. $FeSO_4$ and different amounts of potassium chloride as sole source of potassium. The results may be compared with those shown in the third row of Table 1 (strain A3 on 75 mg. ammonium sulphate) for a culture containing potassium in great excess, and limited by the lactose supply. Similar cultures, giving maximal growth and minimal polysaccharide production, were obtained with potassium supplies down to 1·5 mg. KCl/50 ml. When the amount of potassium was less than this, the amount of growth was decreased in proportion and the amount of polysaccharide production slightly increased. Thus, the results for a medium containing about 0·25 mg. KCl/50 ml. were as follows: final pH 6·5, mucoidness +, average capsule diameter 1·8 μ., intracellular polysaccharide staining +, cell count 58×10^9, wet weight 310 mg., total nitrogen 1·5 mg., deposit polysaccharide 4·8 mg., supernatant polysaccharide 1·1 mg., wet weight:nitrogen ratio 207, polysaccharide:nitrogen ratio 3·7. Further decrease in the potassium supply diminished the amount of growth without increasing the polysaccharide:nitrogen ratio. Thus, potassium deprivation, by limiting growth and preserving the carbohydrate supply, led to an increase of polysaccharide production, but this increase (twofold) was much smaller than the increases (10- to 20-fold) given by equivalent deprivations of the nitrogen, phosphorus or sulphur sources. This suggests that a deficiency of potassium is specifically inhibitory to polysaccharide synthesis in *A. aerogenes* and agrees with the findings of Bernheimer (1953) for washed suspensions of pneumococci.

From the results of this and the previous investigation, the main nutritional requirements of *Aerobacter aerogenes* strain A3 are calculated to be as follows. Growth to give a non-dialysable nitrogen content of 1 mg. (i.e. $30–60 \times 10^9$ cells) requires as a minimum the nitrogen in 5 mg. ammonium sulphate, the phosphorus in 0·45 mg. phosphate (Na_2HPO_4, 3 parts by weight; KH_2PO_4, 1 part), the sulphur in 0·13 mg. sodium sulphate, the potassium in 0·18 mg. potassium chloride, and the carbon and energy of 25 mg. lactose in aerobic conditions.

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EXPLANATION OF PLATE

Figs. 1–3. Colonies of *Aerobacter aerogenes* on MacConkey's agar after 24 hr. at 35°. (×7.) Fig. 1. Mucoid colony of strain A3. Fig. 2. Smooth colony of strain A3 (O). Fig. 3. Rough colony of strain D.301 (R).

Fig. 4. Cells of *A. aerogenes* strain A1 suspended in antiserum for strain A3. Unstained wet film. Shows capsule 'swelling' and agglutination. (×3750.)

Fig. 5. *A. cloacae* strain NCTC 5920 grown on lactose peptone agar for 14 days at 15–20°. In wet film with indian ink. Bacilli with indefinite capsules and much slime (light areas). (×2000.)

Figs. 6–8. *Escherichia coli* strain A55. Intracellular polysaccharide stained by periodate-Schiff method. (×3000.)

Fig. 6. After 4 days at 35° on 250 mg. lactose and 75 mg. ammonium sulphate/50 ml. medium. Minimal polysaccharide staining (⊥).

Fig. 7. After 4 days at 35° on 250 mg. lactose and 7·5 mg. ammonium sulphate/50 ml. medium. Moderate polysaccharide staining (++).

Fig. 8. After 14 days at 15–20° on 250 mg. lactose and 7·5 mg. ammonium sulphate/50 ml. medium. Maximal polysaccharide staining (++++).

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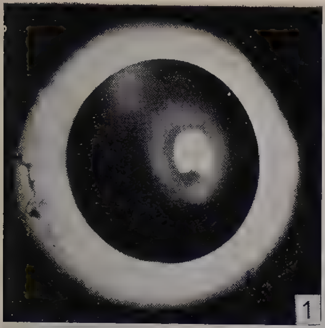
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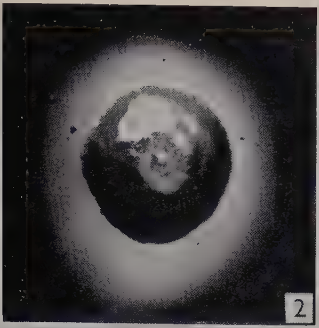
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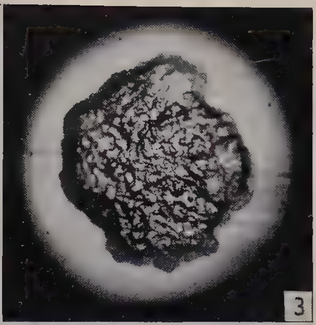
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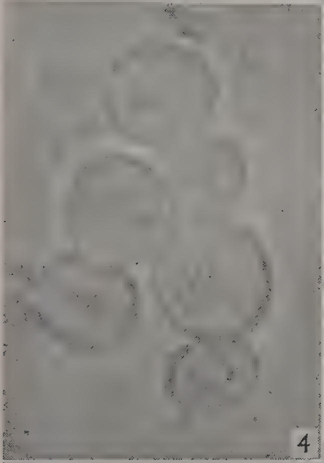
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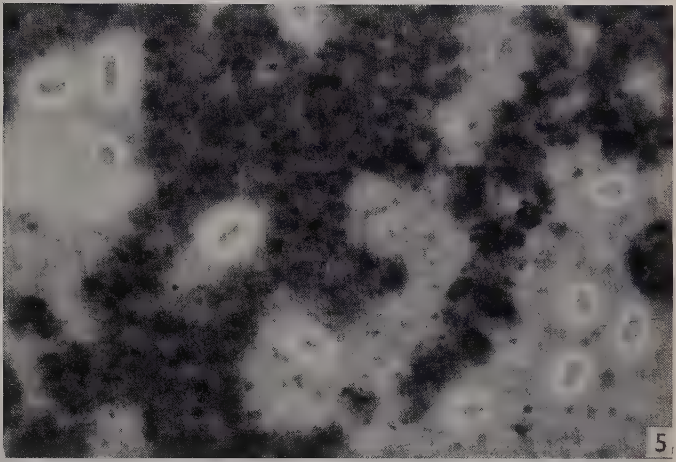
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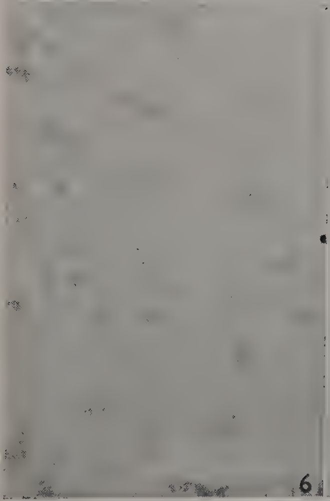
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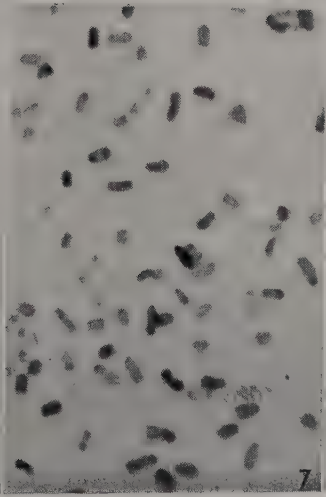
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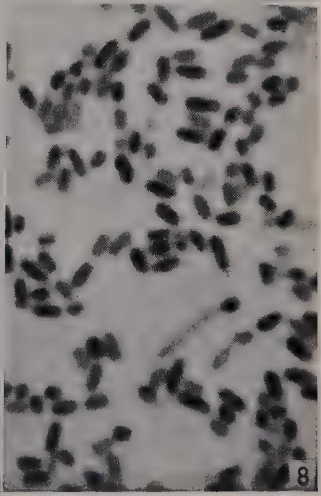
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J. F. Wilkinson, J. P. Duguid & P. N. Edmunds—Polysaccharides of coliform bacteria

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Transport of Phosphate across the Osmotic Barrier of *Micrococcus pyogenes*: Specificity and Kinetics

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SUMMARY: The system coupling inorganic phosphate exchange across the osmotic barrier of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) strain Duncan is highly specific for phosphate, probably for the H_2PO_4' ion. Arsenate, however, can replace phosphate. The exchange is inhibited by thiol reactors, certain anions and certain uncouplers of oxidative phosphorylation. The specificity and kinetics of the exchange suggest that the phosphate is carried in the osmotic barrier as R-phosphate groups moved to and fro across the barrier by thermal agitation, the heat of activation for the movement being 37,400 cal./mole, of which at least 17,700 cal./mole is an entropy component. The exchange of the phosphate of the R-phosphate groups in the barrier with inorganic phosphate on either side may be enzymically catalysed, the free energy of the R-phosphate bond being conserved. Alternatively, the R-phosphate groups may dissociate or hydrolyse, forming free H_2PO_4' ion and R, the latter then being unable to return across the barrier until R-phosphate is again formed by the spontaneous uptake of H_2PO_4' ion.

In resting suspensions of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) strain Duncan, it has been shown that inorganic phosphate molecules can exchange across the osmotic barrier of the cells, but that a net transfer of phosphate across the barrier cannot occur (Mitchell, 1953). It has therefore been suggested that the movement of phosphate molecules across the osmotic barrier is regulated by a carrier system similar to that which Ussing (1947) proposed to account for the exchange-diffusion of cations across a cell membrane. The present paper describes work designed to give more information about the mechanism of exchange of phosphate across the osmotic barrier of *M. pyogenes*. It deals in particular with the specificity of the exchange system for phosphate and with certain aspects of the kinetics of the exchange reaction.

METHODS

Growth and preparation of organism. Cultures of *Micrococcus pyogenes* were grown as previously described (Mitchell & Moyle, 1953): The cells were harvested at a concentration equivalent to 2·5 mg. dry weight/ml., washed twice with distilled water and suspended in distilled water at a dry-weight concentration of *c.* 100 mg./ml. The pH value of the suspensions, measured with a glass electrode, was *c.* 5·5 after washing, it was adjusted to the appropriate value with dilute HCl or NaOH immediately before each experiment.

Estimation of P *and* ^{32}P. The methods used were as previously described (Mitchell & Moyle, 1953).

Estimation of P *in presence of* As. Samples containing 3–30 μg. phosphorus in the presence of up to 75 μg. arsenic could be estimated by adding the Fiske & SubbaRow (1925) reagents in the reverse order, the arsenate thus being reduced to arsenite by the reducing reagent before the addition of the molybdate. To 0·5 ml. samples in stoppered tubes, 0·05 ml. 10 N-H_2SO_4 and 0·2 ml. of the reducing reagent were added. The samples were incubated at 25° for 48 hr., made up to 4·5 ml. with distilled water, and 0·5 ml. of the acid molybdate reagent was added. After 40 min. at 25°, the extinction was determined at a wavelength of 800 mμ. in the Beckman model DU spectrophotometer and compared with standard phosphate + arsenate solutions. The extinction values were reproducible with a standard deviation of ± 1 %, were linear with respect to the phosphate content and independent of the arsenate content of the samples.

Estimation of P + As. Samples containing 0·1–1·0 μmole of arsenate + phosphate could be estimated using stannous chloride in place of the aminonaphtholsulphonic acid reducing reagent in the Fiske & SubbaRow (1925) method. The samples were made up to a volume of 4·3 ml. with distilled water, 0·5 ml. of 2·5 % (w/v) ammonium molybdate in 5 N-H_2SO_4 was added, followed by 0·2 ml. of 0·2 % (w/v) stannous chloride in 0·05 N-HCl. After 40 min. at 25°, the extinction was compared with that of standard phosphate + arsenate solutions as described above. The extinction values were reproducible with a standard deviation of ± 1 % and were linear with respect to both phosphate and arsenate content. The phosphate content of the sample having been determined as described in the previous paragraph, the arsenate content could be obtained by difference.

Manometry. Warburg manometers were used according to the methods recommended by Dixon (1934). The residual fermentation was determined at 25°, using the equivalent of 50 mg. dry-weight cells in 2·5 ml. 100 mM-NaCl containing 2 mM-sodium phosphate and 10 mM-sodium bicarbonate under a gas phase of 95 % (v/v) N_2 and 5 % CO_2, giving a pH value of 7. Fermentation of glucose was measured by tipping in 2·5 μmole glucose in 0·25 ml. water from the side bulb.

Estimation of dry weight. The approximate concentrations of the washed cell suspensions were adjusted turbidimetrically, the exact dry-weight concentrations were measured gravimetrically as previously described (Mitchell, 1953).

Measurement of $\dot{\mathrm{P}}$. The rate of exchange ($\dot{\mathrm{P}}$) of inorganic phosphate across the osmotic barrier was measured in μmole/g. cell dry weight/min. (written μmole/g./min.) by methods similar to those described by Mitchell (1953). The value of $\dot{\mathrm{P}}$ was given by the equation

$$\dot{\mathrm{P}} = \frac{1}{t_2 - t_1} \frac{\mathrm{P_E} . \mathrm{P_I}}{\mathrm{P_E} + \mathrm{P_I}} \ln \left(\frac{1 - R_{t_1}}{1 - R_{t_2}} \right)_{\mathrm{I\ or\ E}}, \tag{1}$$

where R_{t_1} and R_{t_2} represent the relative specific activities of the phosphate with respect to ^{32}P either inside (I) or outside (E) the cells at times t_1 and t_2, and $\mathrm{P_I}$ and $\mathrm{P_E}$ represent the total amounts of phosphate inside and outside the cells.

The routine measurements of $\dot{\mathrm{P}}$ were made at 15° at pH 7 using a cell dry-weight concentration of 20 mg./ml. in suspension media containing 100 mM-

NaCl and 2 mM-phosphate marked with ^{32}P. In experiments where the external phosphate concentration was 10 mM or more, $\dot{P}$ was measured by following the rate of movement of ^{32}P from cells to medium. The time interval t_2-t_1 was adjusted to allow between 20 and 70 % equilibration of the ^{32}P between the internal and external media in all experiments.

RESULTS

Anion specificity and inhibition of phosphate exchange. When added as the sodium salts to give a final concentration of 2 mM (the same as the external phosphate concentration), the following anions had no effect on $\dot{P}$ at 15° at pH 7 and did not cause any change in P_E or P_I: acetate, arsenite, azide, bicarbonate, bromide, chromate, cyanide, ethylenediaminetetra acetate, fluoride, glutamate, glutamine, iodide, molybdate, nitrate, nitrite, oxalate, pyroantimonate, succinate, sulphate, thiosulphate, *p*-toluenesulphonate, tungstate. None of these anions could therefore substitute for phosphate in the system responsible for phosphate exchange. On the other hand, the anions shown below, when present at a final concentration of 1 mM at 15° at pH 7, depressed $\dot{P}$ by the following percentages: permanganate (71), 2:4-dinitrophenate (41), aureomycinate (39), chlorate (30), tetraborate (29), dichromate (26), vanadate (23), sulphite (17), arsenate (17). With the exception of arsenate, however, none of these anions caused any change in P_E or P_I, and could not therefore substitute effectively for phosphate in the exchange reaction.

Table 1. *Movement of* P, ^{32}P *and* As *across the osmotic barrier at* pH 7 *in* 30 *min. at* 15°: *External medium containing* 100 mM-NaCl *and a trace of* $^{32}PO_4$ *with additions shown*

External medium containing	% entry of ^{32}P	% entry of As	% exit of P	$\dot{P}$
2 mM-PO_4	14·0	—	0	0·75
2 mM-PO_4+2 mM-AsO_4	9·5	7·1	7·3	0·48
1 mM-PO_4+1 mM-AsO_4	14·8	10·5	10·7	0·39
2 mM-AsO_4	15·9	11·0	11·1	0

Exchange of phosphate for arsenate. In the suspensions containing arsenate there was a rise in the external phosphate concentration, suggesting that arsenate entered the cells in exchange for phosphate. Moreover, the depression of $\dot{P}$ recorded above for arsenate is about that which would apparently have been given by adding 1 mM-phosphate in place of arsenate and failing to take account of the addition in calculating $\dot{P}$ from equation (1). Exchange experiments were therefore done in which the changes in the amounts of ^{32}P, total P and total As were followed simultaneously in the suspension medium and internal medium of the cells. The total As+P remained constant in both the internal and external media, but the ^{32}P, ^{31}P and As were mutually interchangeable across the osmotic barrier. Table 1 shows a typical set of results. Although $\dot{P}$ was depressed by adding arsenate, the substitution of arsenate for phosphate in the external medium caused the percentage of ^{32}P entering the cells in 30 min. at 15° to increase slightly. Also, when As and ^{32}P were both added to the external medium, the percentage entry of As was always less than that of ^{32}P. It may be concluded that arsenate and phosphate compete in the exchange reaction, and that the probability of reaction of arsenate is somewhat less than that of phosphate under the conditions of these experiments.

Oxidizing agents as inhibitors of exchange. The inhibitory action of permanganate, chlorate, dichromate and vanadate described above might be due to oxidation. Other oxidizing agents were therefore tested. Perchlorate, persulphate, ferricyanide, hydrogen peroxide, iodosobenzoate, benzoquinone, and chlorine did not inhibit phosphate exchange at a concentration of 1 mM, even when incubated with the cells at pH 5 for 15 min. at 25° before $\dot{P}$ was measured at 15° in the usual way. On the other hand, 1 mM-bromine and 1 mM-iodine, although only slightly inhibitory (less than 10 %) after reacting with the cells at pH 7, decreased $\dot{P}$ by 36 and 91 % respectively after reacting with the cells at pH 5·5 for 15 min. at 25°. Since bromine and iodine are more effective inhibitors in acid than in neutral solution, it is probable that they inhibit by oxidation rather than by substitution. However, the inhibitory action of the oxidizing agents appears to depend upon more specific factors than what one would normally regard as their strength as oxidizing agents. The explanation which suggests itself is that the degree of inhibition given by the oxidizing agent depends upon the proportion of its oxidizing capacity which is directed towards the groups essential to the exchange reaction.

Thiol reactors as inhibitors of exchange. In view of the inhibitory action of iodine and bromine described above, and the inhibitory action of mercury and other heavy metals previously described (Mitchell, 1953) the sensitivity of the exchange reaction to substances which alkylate thiol groups was measured. The substances were allowed to react with the cells at pH 7 for 15 min. at 25° before the percentage depression of $\dot{P}$ (shown respectively in the following brackets) was measured at 15° in the usual way: 100 mM-sodium iodoacetate (O), 10 mM- and 100 mM-chloroacetophenone (25 and 70 %), 10 mM- and 100 mM-*N*-ethylmaleimide (13 and 62 %). Evidently, groups which behave like thiol groups of low reactivity are essential to the system which controls phosphate exchange.

Possible triggering of phosphate exchange by residual metabolism. The inhibition of phosphate exchange by the uncouplers of oxidative phosphorylation, 2:4-dinitrophenate and aureomycinate (Loomis & Lipmann, 1948; Loomis, 1950) and by reagents which react with thiols suggest that, although the exchange of phosphate across the osmotic barrier does not in itself involve a net free energy change, a small residual metabolism might be necessary to allow the exchange reaction to occur.

The routine exchange measurements were always done in narrow test-tubes as previously described (Mitchell, 1953) to minimize residual respiration. The suspensions contained 20 mg. dry-weight cells/ml. and it is unlikely that enough oxygen was available for significant respiration except in the top few millimetres of the suspension. However, it was desirable to suppress respiration completely. When this was done by passing a stream of pure nitrogen through cell suspensions in long narrow test-tubes tightly plugged with cotton-wool

$\dot{P}$ remained unchanged. Moreover, $\dot{P}$ was not depressed by 10 mM-KCN, nor was it affected by vigorous aeration of the suspensions. It therefore seemed unlikely that the exchange of phosphate across the osmotic barrier could depend upon residual respiration. It might, however, depend upon residual fermentation.

The residual fermentation was determined at pH 7 at 25° using the equivalent of 50 mg. dry-weight cells. Less than 1 μl. CO_2 (or acid)/hr. was produced. Although the residual fermentation was so low, the cells could rapidly ferment glucose, for the addition of 2·5 μmole glucose caused the production of 110 μl. CO_2, equivalent to 5 μmole acid within 10 min., after which there was no further gas production. This fermentation, however, was completely inhibited when 10 mM-iodoacetate was present. The residual fermentation was thus shown to be so low as to make it unlikely that phosphate exchange could depend upon it. Moreover, even when the system which can ferment added glucose was inhibited by 10 mM-iodoacetate, $\dot{P}$ was unaffected. It therefore seems justifiable that the substances which inhibit the exchange of phosphate across the osmotic barrier do not do so by affecting the residual metabolism, but act directly upon the exchange mechanism. This being the case, kinetic studies should be amenable of interpretation.

Kinetics of phosphate exchange

The effect of external phosphate concentration on $\dot{P}$. The dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) was determined at pH 6·8 in 100 mM-NaCl at 25°. In view of the hyperbolic appearance of the plot of $[P]_E$ against $\dot{P}$, the reciprocals of $\dot{P}$ and $[P]_E$ were plotted and found to give the straight-line relationship of Fig. 1. It is convenient to describe the straight-line relationship in the form

$$1/\dot{P} = 1/\dot{P}_{\text{max.}} + [P\tfrac{1}{2}]_E/[P]_E \cdot \dot{P}_{\text{max.}}, \tag{2}$$

$\dot{P}_{\text{max.}}$ denoting the maximal value of $\dot{P}$ and $[P\tfrac{1}{2}]_E$ denoting the value of $[P]_E$ at which $\dot{P}$ is half maximal. The value of $[P\tfrac{1}{2}]_E$, given by the intercept of the straight line of Fig. 1 on the $1/[P]_E$ axis, is 1·6 mM.

Temperature dependence of $\dot{P}$. The effect of temperature on the relationship between $1/\dot{P}$ and $1/[P]_E$ is shown in Fig. 2. The data represent experiments similar to that of Fig. 1, done at 5° (○) and 15° (●) at pH 6·85. Within experimental error (corresponding to *c.* 0·2 mM-$[P]_E$), the intercept of the straight lines on the $1/[P]_E$ axis is the same as at 25°, and gives a value of 1·6 mM for $[P\tfrac{1}{2}]_E$. Thus, the temperature coefficient of $[P\tfrac{1}{2}]_E$ is practically negligible between 5 and 25°. On the other hand, Fig. 2 shows that $\dot{P}_{\text{max.}}$ (equal to the value of $\dot{P}$ when $1/[P]_E = 0$) has a very large temperature coefficient. Since the temperature coefficient of $[P\tfrac{1}{2}]_E$ is very small, the variation of $\dot{P}_{\text{max.}}$ with temperature is the same as that of $\dot{P}$ at a fixed value of $[P]_E$. It was therefore convenient to determine the temperature dependence of $\dot{P}_{\text{max.}}$ by measuring $\dot{P}$ at an external phosphate concentration of 2 mM in 100 mM-NaCl at pH 7 over a range of temperatures. The results are plotted in Fig. 3 as log $\dot{P}$ against $1/T$, T being the absolute temperature. The points fall on a straight line over the temperature range from 5 to 20°. The slope of this straight line may be represented by the Arrhenius equation, written in the form

$$\frac{d \ln \dot{P}}{d(1/T)} = -\frac{E^*}{R},$$

R being the gas constant and E^*, the activation energy of Arrhenius, having the value 38,000 cal./mole.

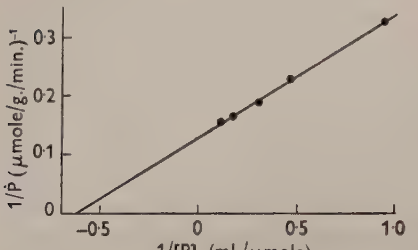

Fig. 1

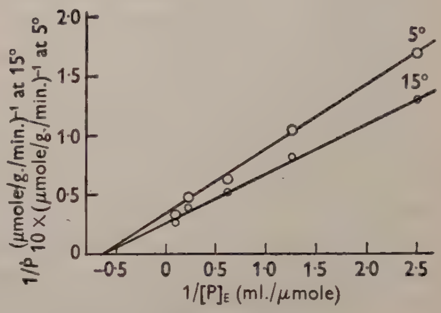

Fig. 2

Fig. 1. Dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) at pH 6·8 in 100 mM-NaCl at 25°.

Fig. 2. Dependence of $\dot{P}$ on the external phosphate concentration ($[P]_E$) at pH 6·8 in 100 mM-NaCl at 5° (○) and 15° (o).

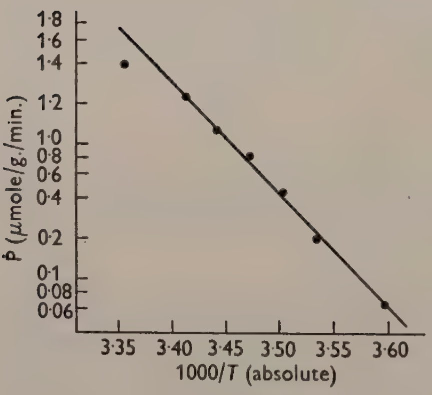

Fig. 3

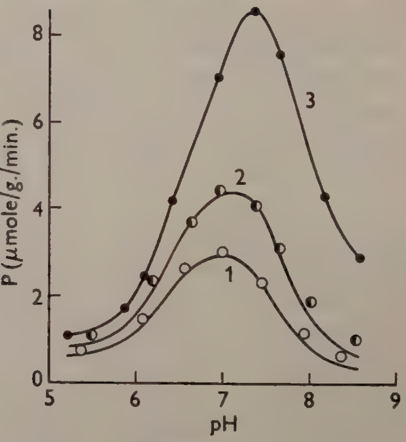

Fig. 4

Fig. 3. Dependence of $\dot{P}$ (logarithmic scale) upon temperature at pH 7 in 100 mM-NaCl containing 2 mM-phosphate.

Fig. 4. Dependence of $\dot{P}$ upon pH at 25° in external media containing 100 mM-NaCl with 1 mM-phosphate (○) or 2 mM-phosphate (◐) or 10 mM-phosphate (●). Curves 1 and 2 were calculated for 1 and 2 mM-phosphate respectively from curve 3, drawn through the points for 10 mM-phosphate.

pH *dependence of* $\dot{P}$. The values of $\dot{P}$ at 25° in external media containing 100 mM-NaCl and 1, 2 and 10 mM-($Na_2HPO_4 + NaH_2PO_4$) over a pH range from 5 to 9 are shown in Fig. 4. The points representing the measurements in 10 mM-phosphate (●), through which the upper curve is drawn, give an optimum value of $\dot{P}$ at pH 7·35, while in 2 mM-phosphate (◐) and 1 mM-phosphate (○) the points in the alkaline range are depressed more than those in the acid range and the optimum value of $\dot{P}$ moves across pH 7. This suggests that the H_2PO_4' ion might be more reactive in the exchange system than the HPO_4'' ion. The pK_2' of phosphoric acid in 100 mM-NaCl is pH 6·8 at 25°, having only a small temperature coefficient (Harned & Owen, 1943), and the value 1·6 mM for $[P\tfrac{1}{2}]_E$ therefore represents 0·8 mM-H_2PO_4' + 0·8 mM-HPO_4''. Assuming that only the H_2PO_4' ion reacts in the exchange system, equation (2) may be written

$$1/\dot{P}+1/\dot{P}_{\text{max.}}+0{\cdot}8/[H_2PO_4']_E\,.\,\dot{P}_{\text{max.}}, \tag{3}$$

expressing $[H_2PO_4']_E$ in mM. From this equation and from the dependence of $[H_2PO_4']$ upon pH and $[P]_E$, namely

$$\text{pH}=6{\cdot}8+\log_{10}\frac{[P]_E-[H_2PO_4']_E}{[H_2PO_4']_E}, \tag{4}$$

the change of $\dot{P}$ with $[P]_E$ at any given pH can be calculated. Thus, the curves 1 and 2 of Fig. 4, representing the values of $\dot{P}$ at $[P]_E = 1$ mM and $[P]_E = 2$ mM respectively, have been calculated from the curve (3) drawn through the points representing $\dot{P}$ at $[P]_E = 10$ mM. The curves approximate reasonably well to the experimental values, and it follows that the data of Fig. 4 are adequately explained if only the H_2PO_4' ion reacts in the exchange system and $[P\tfrac{1}{2}]_E = 0{\cdot}8 \pm 0{\cdot}1$ mM-H_2PO_4', independent of pH. The falling alkaline arm of the pH curves of Fig. 4 is accordingly attributed to the depletion of the H_2PO_4' concentration with increasing pH, and consequent desaturation of the exchange system. The acid arm of the pH curve, on the other hand, shows a dependence of $\dot{P}_{\text{max.}}$ on pH. It follows that, at a phosphate concentration sufficiently high to keep the exchange system nearly saturated with H_2PO_4' ion, the dependence of $\dot{P}$ on pH should nearly represent that of $\dot{P}_{\text{max.}}$ on pH. In an external medium containing 100 mM-phosphate without added NaCl, at 25°, the dependence of $\dot{P}$ on pH is described in Fig. 5. The application of equations (3) and (4) indicates that up to pH 7, $\dot{P}_{\text{max.}}/\dot{P}$ is not greater than 1·02, but at pH's 7·5, 8·0, 8·5 and 9·0 the values of $\dot{P}_{\text{max.}}/\dot{P}$ are 1·05, 1·13, 1·41 and 2·10 respectively. Hence, even at an external phosphate concentration of 100 mM, the falling alkaline arm of the pH curve may probably be attributed to desaturation of the exchange system with H_2PO_4' ion, the value of $\dot{P}_{\text{max.}}$ increasing with pH over the whole range from pH 5 to 9.

DISCUSSION

The system which couples the exchange of phosphate across the osmotic barrier of *Micrococcus pyogenes* is highly specific for phosphate: it behaves as if it were specific for H_2PO_4' ion, the other ionic species of orthophosphate apparently being unable to react. These facts suggest that the phosphate molecules pass across the barrier in combination with a specific carrier group belonging to one of the components of the osmotic barrier. Equation (3) shows that the rate of exchange of the phosphate ($\dot{P}$) depends upon the external concentration of H_2PO_4' ion ($[H_2PO_4']_E$) as though it were a linear function of the degree of saturation with H_2PO_4' ion of a carrier group which is moved to and fro across the barrier by thermal agitation with a frequency independent of $[H_2PO_4']_E$. The value of $[H_2PO_4']_E$ at which $\dot{P}$ is half maximal ($0{\cdot}8 \pm 0{\cdot}1$ mM) would thus correspond to the apparent dissociation constant of the carrier group-phosphate compound, and the maximum value of $\dot{P}$ would correspond to the effective rate of shuttling of the carrier group. If the carrier group (R) were in equilibrium with free H_2PO_4' ion and R-phosphate compound on both sides of the osmotic barrier, and both R and R-phosphate could shuttle across the barrier, unsaturation of R on one side of the barrier would result in a net movement of phosphate into the medium on the unsaturated side. However, even when $[H_2PO_4']_E$ was as low as 0·08 mM (an order of magnitude less than the apparent dissociation constant of the R-phosphate compound), no net transfer of phosphate could be observed. Thus, if the phosphate were to react directly with the R group, it must be supposed that R could move across the barrier only as R-phosphate. Alternatively, R might always be present as R-phosphate in company with a transferring enzyme which catalyses the reaction

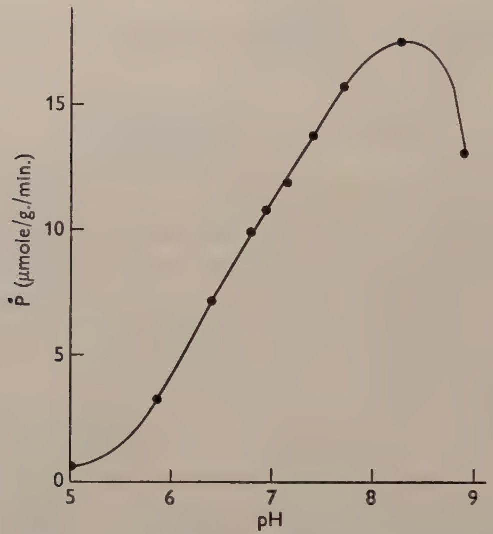

Fig. 5. **Dependence of $\dot{P}$ on pH at 25° in an external medium containing only 100 mM-phosphate.**

$$\text{R-phosphate} + H_2^{*}PO_4' \rightleftharpoons \text{R-}^{*}\text{phosphate} + H_2PO_4'$$

on either side of the osmotic barrier (* being a marker for phosphorus). In this case, the value of $[H_2PO_4']_E$ at which $\dot{P}$ is half maximal would represent the apparent Michaelis constant of the enzyme-H_2PO_4' complex. It will be noted that the exchange of phosphate across the osmotic barrier of *M. pyogenes* is too strictly coupled to be accounted for by the simple exchange-diffusion model proposed by Ussing (1949) to explain cation exchange across the muscle membrane.

It has been observed in this and in an earlier paper (Mitchell, 1953) that the phosphate exchange is specifically inhibited by reagents known to attack thiol groups of low reactivity. This does not prove that the groups attacked are thiols, but it demonstrates that the exchange reaction is dependent upon the integrity of groups with specific chemical properties. These groups might either be the R groups of the carrier compound which react with the H_2PO_4' ion, or they might be groups essential to the activity of the enzyme postulated to catalyse the exchange of H_2PO_4' with the phosphate on R-phosphate.

The temperature coefficient of $\dot{P}_{max.}$ at pH 6·85 represents an Arrhenius energy of 38,000 cal./mole over the temperature range from 5 to 20°. The heat of activation of the exchange process (equal to the Arrhenius energy $-RT$) is therefore 37,400 cal./mole. This heat of activation (ΔH^*) represents the increase in total heat required for the movement of R-phosphate across the osmotic barrier in the two alternative systems proposed above. Measurements of the inhibition of phosphate exchange with phenyl-HgCl (Mitchell, 1953) have shown that the number of sites which would have to bind phenyl-Hg^+ to give complete inhibition is 3% of the number of internal inorganic phosphate molecules, or *c.* 4 μmole/g. This figure gives an upper limit to the number of units operating in the exchange system, and makes possible the calculation of a minimum figure for the absolute reaction rate. The value of $\dot{P}_{max.}$ at pH 6·85 at 20° was *c.* 4 μmole/g./min., and the absolute rate is therefore at least $4/4 = 1$ min.$^{-1}$. In other words, one phosphate group is carried across the osmotic barrier in each direction at least once a minute by each unit of the exchange system. This implies that in the carrier systems proposed above, the R-phosphate group moves across the barrier at least twice a minute. The relationship between the absolute reaction rate and the free energy of activation (ΔF^*), described by Glasstone, Laidler & Eyring (1941) can be written

$$\Delta F^* = RT(\ln (kT/h) - \ln \dot{P}),$$

ln standing for natural logarithm, k for Boltzman's constant and h for Planck's constant. Substituting a value of at least 1 min.$^{-1}$ for $\dot{P}$ (since the energy required to activate the movement of the carrier across the barrier in one direction may be much greater than that in the other direction), the free energy of activation for the movement of the R-phosphate group across the barrier is found to be not more than 19,700 cal./mole. Since $\Delta H^* = \Delta F^* + T\Delta S^*$, a large part (at least 17,700 cal./mole) of the heat of activation (37,400 cal./mole) may be attributed to the entropy change (ΔS^*) as in reactions like the denaturation of proteins (Glasstone, Laidler & Eyring, 1941). The thermodynamic data are thus in accord with the view that the osmotic barrier, across which the carrier effects the movement of phosphate and of which the carrier forms an integral part, is a well organized internally bonded structure, the disturbance caused by the movement of the carrier within the barrier being analogous to reversible protein denaturation.

It was suggested by Mitchell & Moyle (1951) that the lipo-protein particles which can be obtained from disintegrated *Micrococcus pyogenes* represent fragments of the cell membrane which, in the intact cells, would form a sheet *c.* 15 mμ. thick. It is possible that this sheet represents the osmotic barrier for phosphate and that the system responsible for phosphate transport may be found in the lipo-protein particles.

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Studies of some Presumptive Lactobacilli isolated from the Rumens of Young Calves

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SUMMARY: Ten out of thirteen isolates of Gram-positive rods from the rumens of young calves proved to be catalase-negative and were further studied by methods suitable for lactobacilli. Four isolates from two calves, one of which was aureomycin-fed, proved to be *Lactobacillus brevis*. Three isolates from a very young calf not fed aureomycin were provisionally identified as an anaerobic variant of *L. lactis*. The remaining three isolates obtained from an aureomycin-fed calf were all motile, homofermentative and mannitol-fermenting, and could not be identified with any known species of *Lactobacillus*; this organism produced a mixture of dextrorotatory and inactive lactic acid from glucose. The fermentation reactions of all the presumed lactobacilli isolated in this study were clear-cut and reproducible.

Mann, Masson & Oxford (1954) fed aureomycin (40–60 mg.) once daily to calves from the 2nd to the 15th week of age, but on slaughter could detect the antibiotic only in the abomasum, never in the rumen. Furthermore, the only noteworthy bacteriological differences between the rumen microflora of aureomycin-fed and control calves were that the rumen of the youngest control calf contained a considerable population of anaerobic Gram-positive rods not found in two aureomycin fed calves of the same age, and that amylolytic streptococci were rather more plentiful in the rumens of older control calves than in those of aureomycin-fed animals.

This paper is devoted to a description of thirteen isolates of Gram-positive rods (including three anaerobes) obtained in the foregoing study; most of them seemed to be lactobacilli. The accompanying Gram-positive cocci (sixty-nine isolates) and facultatively anaerobic Gram-negative rods (forty isolates) will be described later.

METHODS

Feeding of calves. This is fully described by Mann *et al.* (1954). In this connexion the relevant points are that fresh unpasteurized T.T. milk was fed up to the 6th week of age, and that the aureomycin was administered not in the milk but dissolved in an aqueous gruel.

Isolation of rumen bacteria in pure culture. Unless otherwise stated, all incubations were done at 38°. Bottle counts on rumen contents immediately after slaughter of the calf were carried out by the method of Heald, Krogh, Mann, Appleby, Masson & Oxford (1953) devised for sheep rumen contents. The basal agar contained absolutely clear sterilized sheep rumen liquor since it was not practicable to prepare clear calf rumen liquor on the scale required. As mentioned by Mann *et al.* (1954) both cellobiose and mannitol were employed as carbohydrate substrates. These authors also listed the actual viable counts obtained, which were never very large, i.e. more than 10^8/g., save with the first (1 C) and last (3 C) control calves, aged 32 and 86 days respectively. Pure culture isolations were made from all bottles which contained *c.* 10–50 clearly visible and well-separated colonies after 3 days' incubation at 37°. Except with the first control calf (1 C; 32 days old) these proved mostly to consist of Gram-positive cocci or small Gram-negative rods. Cultures of Gram-positive rods were, however, also obtained from the rumens of each of the following three calves (out of a total of eight, excluding 1 C): 1 A (aureomycin-fed, 33 days old); 2 A (aureomycin-fed, 54 days old) and 3 A^1 (aureomycin-fed, 96 days old). It should be emphasized that the initial isolation was not made from an agar medium especially devised for the isolation of lactobacilli. Consequently, it is unlikely that any exacting milk lactobacilli, the growth requirements of which might not be met by rumen liquor, would be detected in our counts, unless of course they were making use of growth factors synthesized by other bacteria forming colonies in the same bottle.

The cultures of rumen Gram-positive rods were purified by plating from glucose yeast-extract peptone broth on to agars of the same composition, which were incubated both aerobically and anaerobically. It was found that Robertson's cooked meat medium was not suitable for the maintenance of stock cultures, which were therefore kept in the freeze-dried state wherever possible.

Bacteriological methods. These followed those of Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953) very closely, since we were primarily concerned with the fermentation reactions of our organisms, and these authors had claimed almost absolute reproducibility under their conditions of test. Gas production from glucose (distinction between homofermentative and heterofermentative lactobacilli) was observed in the complex tomato juice agar stab cultures recommended by these authors. Fermentation tests were carried out in duplicate exactly as recommended, with washed suspensions from 24 hr. tomato-broth cultures as inocula (48 hr. cultures for the anaerobes). The whole series of fermentation tests was repeated several weeks later in order to test their reproducibility.

RESULTS

Origin of cultures of rumen Gram-positive rods. Such cultures were isolated from both cellobiose- and mannitol-containing bottles used in the count. Table 1 shows the several highest dilutions of rumen contents at which Gram-positive rods were isolatable. They were always in evidence in direct rumen smears, particularly from the younger calves. Of the thirteen isolates listed in Table 1 only nos. 1–10 were catalase-negative and showed no ability to reduce nitrate. Isolates 11, 12 and 13 (from the oldest calf) were catalase-positive and reduced nitrate; although they grew quite well at 38° under anaerobic conditions and did not seem to form spores, obviously they did not belong to the genus *Lactobacillus* as usually defined, and were therefore not studied further.

On the basis of morphology and fermentation reactions the first ten isolates were found to fall into three groups only, all isolates in each group being virtually identical. These three groups were clearly differentiated from each other and are regarded as representing three separate and distinct rumen organisms, namely organism 123 (comprising isolates 1–3), organism 678 (comprising isolates 6–8) and organism 45910 (comprising isolates 4, 5, 9 and 10) respectively. It will be seen from Table 1 that the first and second organisms were each derived from one calf only (although in the second instance both mannitol and cellobiose bottles provided isolates), whereas the last organism (45910) comprised two isolates from each of two calves, one of which was fed aureomycin and the other not.

Table 1. *Origins of cultures of rumen Gram-positive rods*

Calf no.	Age of calf (days)	Isolate no.	Highest dilution and carbohydrate used in bottle count	Whether Gram-positive cocci also isolated from same bottle
1 C	32	1	*c*:CL	No
		2	*a*:CLV	No
		3	*a*:CLV	
		4	*a*:M	Yes
		5	*a*:M	
1 A	33	6	*b*:C	Yes
		7	*b*:M	Yes
		8	*b*:M	
2 A	54	9	*c*:C	Yes
		10	*c*:C	
3 A^1	96	11	*a*:M	Yes
		12	*a*:M	
		13	*a*:M	

Calves: A or A^1, aureomycin-fed; C, control, not fed aureomycin (see Mann, Masson & Oxford, 1954).

Dilutions of rumen contents: $a = 1/10,000$; $b = 1/500,000$; $c = 1/3,000,000$.

Carbohydrates: CL, cellobiose; CLV, cellobiose + crystal violet (0·0005 %); M, mannitol.

Properties of organism 123

This organism was obtained only from the rumen of the first control calf. It formed colonies only in the cellobiose nutrient agar used for the viable bacterial count, not in the corresponding medium containing mannitol. It was an obligate anaerobe and in the bottle count grew below the surface of the agar to the exclusion, at the higher dilutions, of all other visible colonies either above or below the line of demarcation of the growth. A very similar picture was presented when the organism was grown in pure culture in glucose tomato-extract agar. In liquid medium, likewise, there was no growth in any instance unless the tube was incubated in an anaerobic jar. The organism seemed to have no special CO_2 requirement. On blood agar or nutrient agar plates, incubated anaerobically, the organism formed rough colonies, 1·5 mm. in diameter, with undulating edge. The organism grew well at 45° but not at 15°. None of the three isolates was aureomycin resistant.

Morphology. Films made from fluid cultures of organism 123, at all ages, and stained by Gram's method showed short and long (unbranched) filamentous rods, often highly curled and several hundred microns in length. These long filaments were not seen in direct smears of rumen contents. The staining was uneven and showed granulation. Gram-negative elements were also in evidence in older cultures, although the predominant appearance was always of Gram-positive rods and filaments. Spores were never observed; the organism was non-motile.

Biochemical reactions. Organism 123 was weakly and inconsistently heterofermentative by the test of Rogosa *et al.* (1953). It produced acid followed by coagulation in litmus milk in 4 days at 38°.

The following carbohydrates were fermented with production of an acid pH value (numerical value in brackets) in each instance: aesculin (4·3); cellobiose (4·2); dextrin (4·6); fructose (4·1); galactose (4·3); glucose (4·2); lactose (4·2); maltose (4·2); mannose (4·2); melibiose (4·5); raffinose (4·3); salicin (4·4); sucrose (4·3); trehalose (4·2). The following compounds were not fermented, i.e. the pH value of the medium remained constant or fell only one or two tenths of a unit from the original value (5·9) even when growth occurred: arabinose, adonitol, dulcitol, glycerol, inositol, inulin, mannitol, melezitose, rhamnose, sorbitol, starch, xylose. All three isolates of organism 123 behaved exactly similarly in fermentation tests, which, when repeated several weeks later, gave the identical picture shown above.

In a fermentation of 500 ml. of a 2 % glucose tomato-extract broth, the pH value fell to 4·1 in a few days at 37°, giving a copious white bacterial deposit (*c.* 0·3 g. when dried), with an absolutely clear supernatant from which the zinc salt of laevorotatory lactic acid was easily isolated by the standard method. In our opinion Briggs (1953) overestimates the difficulty of carrying out this important test, the sign of rotation of the lactic acid being of considerable diagnostic value. It is important, however, actually to determine the percentage of lactate in the crude zinc salt so isolated, by a specific method such as that of Barker & Summerson (1941), since malic acid, which also can exhibit optical activity, is a possible fermentation product with lactobacilli. Even so, the total time consumed in these procedures is not great; about two working days. No zinc lactate could be isolated from the unsown medium when put through the same process.

Organism 123 did not hydrolyse hippurate or liquefy gelatin. It did not produce a soluble haemolysin.

Pathogenicity. Non-pathogenic to mice when injected intramuscularly or intraperitoneally; no local lesions.

Properties of organism 678

This was a facultative anaerobe; it yielded translucent colonies 0·75–1·0 mm. diameter, with raised centre and undulating edge with wavy projections after 2 days' incubation on nutrient agar. When grown in poured glucose agar deeps incubated aerobically, smooth colonies were formed at all levels. The organism grew profusely in glucose tomato-extract medium with good turbidity and sediment. Motility was observed after 16 hr. with all three isolates; this observation was repeated many times. The motility, although sluggish, was unmistakable even in a liquid culture which filled a 500 ml. conical flask with a narrow neck. None of the isolates (6–8) was aureomycin-resistant. The organism grew well at 45° but not at all at 15°.

Morphology. Films made from fluid cultures and stained by Gram's method showed straight and curved Gram-positive rods with rounded ends ranging from 2 to 7 μ. in length, diameter 0·75 μ., together with some longer filaments up to 48 μ. long. Staining was variable and some Gram-negative elements were observed in cultures of any age. Spores were never seen.

Biochemical reactions. The organism was homofermentative by the test of Rogosa *et al.* (1953); it acidified and coagulated litmus milk. The following carbohydrates were fermented with production of an acid pH value (numerical value in brackets) in each instance: aesculin (4·3), cellobiose (4·0), dextrin (4·6), fructose (3·9), galactose (4·1), glucose (3·9), lactose (4·1), maltose (4·0), mannitol (4·0), mannose (4·1), raffinose (3·9), salicin (4·1), sorbitol (4·1), sucrose (4·0), trehalose (3·8), xylose (5·0–5·4), melibiose (4·1). The following compounds were not fermented: arabinose, adonitol, dulcitol, glycerol, inositol, inulin, melezitose, rhamnose and starch. The organism thus has wider fermentative powers than the anaerobic organism 123, although the fermentation of xylose was weak. Repetition of the whole series of fermentation tests yielded exactly the same results.

In a fermentation of 500 ml. of a 2 % glucose tomato-extract broth the pH value fell to 4·0 in 6 days at 37°, giving a much greyer bacterial deposit (after centrifuging) than with the anaerobic organism 123, although the yield of dried cells was only slightly less. Inactive and dextrorotatory lactic acid were the only fermentation products. No volatile acid was produced.

Organism 678 did not hydrolyse hippurate or liquefy gelatin. It produced distinct α-haemolysis of sheep red cells when grown on blood-agar plates.

Pathogenicity. Non-pathogenic to mice, as organism 123.

Properties of organism 45910

This was a facultative anaerobe, but it did not grow as profusely in liquid media or on nutrient or (particularly) blood agar as did organism 678. The colonies were 0·5–1·0 mm. diameter, smooth, translucent with entire edge and raised centre. All four isolates (4, 5, 9 and 10) grew better under microaerophilic conditions, or in an atmosphere containing 10 % CO_2. In liquid media growth was chiefly granular with some turbidity but no pellicle. The organism was non-motile. None of the isolates was aureomycin-resistant. The organism grew well at 45° but not at all at 15°.

Morphology. This was very similar indeed to that of organism 678; the rods in general were rather thicker, of diameter 1·0 μ.

Biochemical reactions. Organism 45910 was strongly heterofermentative (distinction from organisms 123 and 678). It acidified and coagulated litmus milk. The following carbohydrates were fermented with production of an acid pH value (numerical value in brackets) in each instance: arabinose (4·0), aesculin (4·8), fructose (4·7), galactose (4·4), glucose (4·4), lactose (4·5), maltose (4·5), melibiose (4·5), raffinose (4·6), salicin (4·9), sucrose (4·7), xylose (4·0). The following compounds were not fermented: adonitol, cellobiose, dextrin, dulcitol, glycerol, inositol, inulin, mannitol, mannose, melezitose, rhamnose, sorbitol, starch, trehalose. Unlike organisms 123 and 678, organism 45910 is a powerful pentose-fermenter, although with hexoses, as befits a heterofermentative lactobacillus, it does not yield as low a pH value in the medium as these other non- or weakly heterofermentative strains. Repetition of the whole series of fermentation tests yielded exactly the same results. The lactic acid produced from glucose was the inactive variety.

Organism 45910 did not hydrolyse hippurate or liquefy gelatin. It produced no haemolysin for sheep red cells.

Pathogenicity. Non-pathogenic to mice, as organism 123.

DISCUSSION

No claim is made that all the lactobacilli or related Gram-positive rods in the rumens of these young calves have been isolated. We have merely isolated those which grew well in a solid medium suitable for the isolation of typical rumen streptococci. However, we regard it as significant that no Gram-positive catalase-negative rods were isolated from the third group of calves which were killed some weeks after the discontinuance of milk in their ration. This probably means that no lactobacilli from milk become really established in the calves' rumen: they are merely organisms of passage, capable sometimes of multiplying greatly for a while in the rumen of a very young calf (e.g. our 1C aged 32 days), but not persisting for long after their source is withdrawn. It is probably not to the advantage of the calf that lactobacilli should multiply greatly in its rumen for, of all saccharolytic bacteria, the homofermentative lactobacilli most quickly produce a low pH value and thereby effectively stop the development of 'true' rumen bacteria which flourish best between pH 6 and 7. Our calf 1C, which had a large population of an anaerobic lactobacillus in its rumen, also had a low rumen pH value of 5·0.

With regard to the identification of our three catalase-negative rod organisms, the strongly heterofermentative organism 45910 (represented by four isolates from two different calves, one aged 33 and the other 54 days) presents the least difficulty, since its biochemical reactions agree very well with those of *Lactobacillus brevis* (see group 1 in table 5 of Rogosa *et al.* 1953). Its ability to hydrolyse aesculin would appear to exclude *L. fermenti*. The only important difference between our organism and the organism of Rogosa *et al.* is that ours does not hydrolyse hippurate. The obligate anaerobic organism 123 is more difficult to place. It might well pass for a homofermentative lactobacillus unless the test for gas production is very carefully carried out, but it cannot be the well-known homofermentative anaerobe *L. bifidus*, since branching was never observed and it produced laevo- instead of dextrorotatory lactic acid. Its morphology and fermentation reactions agree quite well with the homofermentative *L. lactis* (see *Bergey's Manual*, 1948, p. 351) which is said to produce long filaments and threads and to ferment the same sugars as did our organism 123, which also produces laevorotatory lactic acid. Organism 123 might therefore be regarded as an anaerobic variety of *L. lactis*. Our third organism (678), although a homofermentative lactic-acid producing rod, cannot be identified with any known organism. It may not belong to the genus *Lactobacillus* at all since it is motile, ferments mannitol and does not grow at 16°. Although no known species of homofermentative *Lactobacillus* has these characteristics (see table 1 in Rogosa *et al.* 1953; *Bergey's Manual*, 1948), it is clearly difficult to assign organism 678 to any other genus. Until the question of the motility of lactobacilli is cleared up, we hesitate to assign a new species name to this strain. It is clearly very similar in many respects, including fermentation reactions and the kind of lactic acid produced, to the motile lactobacillus isolated by Harrison & Hansen (1950) from the caecal faeces of turkeys.

Two points of general interest emerge from this study. First, it is evident that the direct morphological approach to rumen microbiology, as advocated by Baker & Harris (1947–8) and Moir & Masson (1952), although undeniably useful, has its limitations for it takes no account of the extreme pleomorphism encountered in genera like *Lactobacillus*, certain members of which, from their biochemical and physiological reactions, might conceivably flourish in the rumen under certain conditions. Our organism 123, for example, consisted in pure culture mostly of filaments, whereas in direct rumen smears only the short rod-forms were in evidence. There can be little doubt, however, that organism 123 did in fact occur in great numbers (*c.* 10^9/g.) in the rumen contents of calf 1 C (see Table 1). Finally, our admittedly limited experience with the fermentation reactions of lactobacilli is more in accord with the findings of Rogosa *et al.* (1953) who claim reproducibility, than with the conclusions of Briggs (1953), who made no use of these reactions for classificatory purposes on account of their alleged unreliability. Since lactobacilli are carbohydrate fermenters *par excellence* it surely would be advantageous to use their fermentation reactions for classificatory purposes, as with streptococci, if reproducibility can be assured. The basal medium of Rogosa *et al.* seems to us to be a definite step forward to this desirable end.

We are grateful to our colleagues: Miss Elizabeth Mackay for the isolation of two anaerobic isolates on the crystal violet medium, and Dr R. J. Pennington for confirming, by the Barker-Summerson method, that our crude fermentation zinc lactates consisted in every case essentially of that salt.

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A Selective Medium for the Primary Isolation of *Haemophilus pertussis* and *Haemophilus parapertussis*

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SUMMARY: A description is given of a selective medium which supports the growth of *Haemophilus pertussis* and *Haemophilus parapertussis* and largely inhibits the growth of *Haemophilus influenzae.* The medium contains horse-meat extract, horse blood, salt agar, 2 μg. 4:4′-diamidino-diphenylamine-dihydrochloride/ml. and 0·3 unit penicillin/ml. The medium is of value in the primary isolation of *H. pertussis* and *H. parapertussis.*

Dawson, Farnworth, McLeod & Nicholson (1951) reported that a medium containing beef extract and horse blood was able to support the growth of *Haemophilus pertussis* while considerably inhibiting the growth of *H. influenzae.* This inhibitory effect was shown to be associated with the horse blood, and we have since shown that an even greater inhibitory effect can be obtained by using horse-meat extract instead of beef extract. During the course of this work Lacey (1951) described a medium which was highly selective for *H. pertussis* containing 0·3 units penicillin/ml. and 12 μg. 4:4′-diamidino-diphenylamine-HCl/ml. In view of Lacey's findings we investigated the possibility of incorporating 4:4′-diamidino-diphenylamine-HCl in our medium. It was shown that the growth of *H. influenzae* was completely suppressed by one-sixth of the concentration of the diamidine used by Lacey, while the growth of *H. pertussis* and *H. parapertussis* was practically unaffected. As a result of this finding we prepared a meat extract medium containing the diamidine and penicillin and tested its value for the primary isolation of *H. pertussis.*

EXPERIMENTAL

Bordet–Gengou medium

A mixture of 100 g. thinly sliced potato, 8 g. glycerol and 192 ml. distilled water was steamed for 2 hr. and filtered through lint. The extract was added to 8 g. agar (Davis Standard—from Thomson, Skinner & Hamilton Ltd., 12 Cadogan Street, Glasgow, C.2) dissolved in 600 ml. 0·6 % NaCl and sterilized by autoclaving at 10 lb./sq.in. for 15 min. Finally 45 ml. of defibrinated rabbit blood and 0·9 ml. of a solution of penicillin G (50 units/ml.) were added to each 100 ml. of the basal medium.

Meat-extract medium

Lean horse meat (best steak; 500 g.) was minced and steamed for 2 hr. with 1 l. distilled water containing 6 g. NaCl. The extract was filtered hot through filter-paper (Whatman no. 1) and to the clear fat-free filtrate (1000–1050 ml.), 10 g. agar (Davis) was added. The pH was adjusted to 7·8 and the basal medium so obtained sterilized by autoclaving at 10 lb./sq.in. for 15 min. After cooling to 50°, 25 ml. oxalated horse blood (Burroughs Wellcome & Co.), 0·75 ml. of penicillin G. solution (50 units/ml.), and 0·3 ml. of a 0·1 % (w/v) solution of 4:4′-diamidino-diphenylamine-dihydrochloride (M. & B. 938) were added to each 100 ml. of basal medium.

It is important in the preparation of this medium to ensure that it is free from fat which is inhibitory to *Haemophilus pertussis*. The hot filtration through filter-paper is usually adequate if all gross fat is removed from the meat before mincing. If, however, the filtrate does contain fat globules it should be allowed to stand overnight at about 4° and the solidified fat particles then removed by a further filtration.

RESULTS

Two hundred and sixty-four pernasal swabs from children suspected of whooping cough were cultured on Bordet–Gengou medium (BG) and on the selective meat-extract medium (ME) in parallel. At first each swab was inoculated on a $2\frac{3}{4}$ in. diam. Petri dish of each medium, and these dishes were used throughout for BG medium. The greater selectivity of the ME medium, however, made it possible in the later stages of the work to use only one-half of a $2\frac{3}{4}$ in. plate or one-quarter of a $3\frac{1}{2}$ in. plate without any apparent loss of efficiency. After inoculation the plates were incubated for 4 days at 37°.

From 264 pernasal swabs examined in this way, *Haemophilus pertussis* was isolated on thirty-eight occasions on BG, and fifty-two occasions on ME. Growth of organisms other than *H. pertussis* was obtained on 49 % of the BG plates inoculated. With ME medium the growth other than *H. pertussis* was usually confined to a small number of colonies of diphtheroid or coliform organisms. That this greater selectivity was achieved without detriment to *H. pertussis* is suggested by the observation on twenty plates that the average colony diameter of the organism was 0·55 mm. on BG, and 0·60 mm. on ME medium. There was no evidence, however, that growth of *H. pertussis* was more rapid on ME than on BG.

Haemophilus parapertussis was shown to grow quite well on the ME medium, and although isolated only twice during the comparative tests has also been isolated on several occasions on ME when the BG medium was not regularly in use. As with *H. pertussis* growth was observed after 2 or 3 days.

CONCLUSIONS

The advantages of this selective medium containing horse-meat extract, horse blood, salt, penicillin and 4:4′-diamidino-diphenylamine are: (1) it is relatively easy to prepare; (2) only 20 % of sterile oxalated horse blood is required; (3) it is highly selective since significant growth of organisms other than *Haemophilus pertussis* and *H. parapertussis* seldom occurs, and it is not inhibitory to these latter organisms; (4) the percentage of positive isolations exceeds that obtained with the Bordet–Gengou medium; (5) the medium is a reliable one for the isolation of *H. parapertussis*.

We wish to acknowledge the help and advice of Prof. J. W. McLeod, and the technical assistance given by Miss B. B. Waller and Miss J. Cuckston. Most of the pernasal swabs were received from Dr G. R. Baxter of the Leeds Public Health Department, in connexion with the M.R.C. whooping-cough inquiry.

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Parasexual Recombination in *Penicillium chrysogenum*

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SUMMARY: Roper's (1952) technique for the isolation in filamentous fungi of strains carrying in their hyphae diploid nuclei heterozygous for known markers has been applied with minor modifications to a third species: *Penicillium chrysogenum*. One of these modifications is the use of 'dwarf' (stunted growth) mutants for growing balanced heterokaryons. Heterozygous diploid strains of *P. chrysogenum* behave like those of the other two species in yielding, vegetatively, new strains with recombined properties. This behaviour can be marshalled for the deliberate breeding of improved industrial strains.

In two species of filamentous fungi, *Aspergillus nidulans* (Roper, 1952; Pontecorvo & Roper, 1952, 1953; Pontecorvo, Tarr Gloor & Forbes, 1954) and *Aspergillus niger* (Pontecorvo, 1952; Pontecorvo, Roper & Forbes, 1953), recombination between properties of different strains may take place outside, or in the absence of, a sexual cycle. Processes leading to gene recombination otherwise than via sexual reproduction are called 'parasexual' (Pontecorvo, 1953*a*). The present paper reports the discovery of a parasexual cycle in a third species, *Penicillium chrysogenum*, the mould used for the industrial production of penicillin. This discovery was the outcome of the application, with minor modifications, to this species of the techniques developed for the other two species.

The differences in properties used for the present work (colours of conidia, growth rates, colony morphology and nutritional requirements) were chosen exclusively for their convenience as experimental markers. The discovery that recombination occurs in respect of these properties makes it certain that it also occurs in respect of inherited properties of other kinds. Clearly, this knowledge makes possible the deliberate breeding of improved strains for penicillin production. A preliminary report of the work in this paper has been published (Pontecorvo & Sermonti, 1953).

The production of marked strains

Unless explicitly stated, the techniques used in the present work are the same as those used as a routine for *Aspergillus nidulans* (Pontecorvo, 1953*b*) and applied to *A. niger* (Pontecorvo *et al.* 1953).

Media. Minimal medium (MM): a modified Czapek-Dox. Complete Medium (CM): a complex medium containing yeast extract, casein hydrolysate, etc. Incubation was at 26°.

Organisms. Two starting strains of *Penicillium chrysogenum* were used; one with white conidia, turning very pale green after long incubation at room temperature, and one with yellow-brown conidia. The former, kindly supplied by Dr J. C. Calam (Imperial Chemical Industries, Manchester) was a mutant obtained after nitrogen mustard treatment. It is designated here with the symbol *w*. The yellow strain (symbol *y*, code no. *1086*) was obtained previously by one of us (G.S.) after ultraviolet irradiation of strain *Q176.47.1564*. Both were prototrophs, i.e. able to grow well on medium MM, like the green-spored strain *Q176*, to which both ultimately traced back.

The marked strains required for the present work were strains differing from the wild type in three pairs of alternative characters and from each other in six pairs. Two pairs (white versus green conidia, yellow versus green conidia) were already available in the two starting strains mentioned above. The other four pairs were obtained by successive ultraviolet irradiations and they consisted either in differences in nutritional requirements or differences in growth rates (e.g. dwarf versus normal colony size).

Table 1. *Results of ultraviolet irradiation* (4 *min. at* 30 *cm.*) *of dishes of CM agar plated with about* 1000 *conidia each*

				Mutants among isolates				
Strain irradiated		Viable counts (%)	Colonies isolated (No.)	Nutritional			Dwarf	
Code No.	Colour and requirements			No.	Code No.	Additional requirements	No.	Code No.
w	White, no requirements	2	238	3	*4w*	NO_2^-	—	—
					7w	Thiosulphate		
					8w	Pyridoxine		
y	Yellow, no requirements	5	160	3	*19y*	Thiosulphate	—	—
					22y	Hypoxanthine		
					38y	NO_2^-		
22y	Yellow, hypoxanthine	17	325	4	*22y 5*	NH_2 groups	1	*22y dw 1*
					22y 13	Methionine		
					22y 14	Arginine		
					22y 15	Anthranilic acid		
7w	White, thiosulphate	6	150	3	*7w 13*	Hypoxanthine	2	*7w dw 4*
					7w 16	Adenine		*7w dw 5*
					7w 32	Hypoxanthine		
38y	Yellow, NO_2^-	0·2	125	2	*38y 2*	Peptone	1	*38y dw 6*
					38y 3	Thiosulphate		
			998	15				

Suspensions of 10^4 conidia/ml. were spread in volumes of 0·1 ml./plate (1000 conidia) over the agar surface of a series of Petri dishes containing CM agar. The dishes were irradiated by exposing them to an Hanovia XI low-pressure mercury lamp for 4 min. at 30 cm. distance. Colonies which grew up (random sample) after such treatment (Table 1) were isolated and screened for mutants with additional growth factor requirements (Pontecorvo, 1953*b*). Furthermore, four dwarf mutants were picked by inspection from some of the irradiation series. The mutants were purified by micromanipulation of single conidia or by plating followed by single colony isolation.

Synthesis of balanced heterokaryons

Previous attempts by one of us (G.P.) to form balanced heterokaryons between pairs of strains each differing from the wild type in one additional nutritional requirement had been unsuccessful. A similar failure is mentioned by Bonner (1946) in respect of pairs of strains of *Penicillium notatum*; yet heterokaryosis in this species (Pontecorvo & Gemmell, 1944; Sansome, 1947) was known to occur. The technique used in our attempts was the one which has invariably proved effective with other species of moulds (Pontecorvo, 1947, 1953*b*). In its most recent version it involves the following steps: (1) inoculation into liquid CM of a thick suspension of about equal numbers of conidia of each of two strains requiring different growth factors; (2) incubation for a time sufficient to obtain a thin mesh of mycelium; (3) removal of this mesh, which is washed by dipping in MM, and thoroughly broken up by teasing out over the surface of plates of MM agar; (4) incubation of these until growth starts from a few points; (5) isolation of growing hyphal tips from these points on to MM agar. The length of incubation (under 4) required for the 'escape' of the heterokaryotic growth varies with the species and with the particular pair of strains used (4 days for most combinations in *Aspergillus nidulans* and in *A. niger*).

The growth rate on agar medium of *Penicillium chrysogenum* is about one-fifth of that of *Aspergillus nidulans*. It was therefore expected that the time of incubation necessary for the escape on MM agar of heterokaryotic growth would be much longer. In fact, in the unsuccessful attempts mentioned above, after 2 or 3 weeks the MM plates would begin to show widespread growth, but this proved to be syntrophic, *not* heterokaryotic. The conditions making it possible for balanced heterokaryotic mycelium to become established to the exclusion of, or in equilibrium with, homokaryotic mycelium have been discussed by Pontecorvo (1953*b*). It appeared likely that the failure just mentioned might be due to the very low growth rate of *P. chrysogenum*. A growth rate small compared to the rate of diffusion of metabolites may not confer on the heterokaryon an advantage over the two syntrophically growing homokaryons sufficient for the heterokaryon to become established.

This kind of reasoning suggested two ways out. First, the use of pairs of strains with a very much decreased growth rate not capable of being restored to normal by the supply of growth factors; the possibility of syntrophism would thus be excluded. Strains of this type constitute an appreciable proportion of those obtained after irradiation and are identified by the tiny colonies they form even on CM agar. They will be referred to as 'dwarf' strains. Balanced heterokaryons with normal growth rate, formed between pairs of dwarf strains, have been described in other species (Dodge, 1942; Pontecorvo, 1947). Dodge's work gave, in fact, the first example of balanced heterokaryosis.

Secondly, the use of pairs of strains with more than one growth factor requirement, in order to make their metabolic interdependence in the absence of the growth factors more interlocked, and therefore presumably favouring intracellular (heterokaryotic) rather than intercellular (syntrophic) co-operation. This metabolic interlocking might be particularly effective when the growth factor requirements of the two strains are metabolically related in pairs, as in the case of different blocks on the same pathway of synthesis; e.g. requirement of adenine or hypoxanthine versus adenine only; for methionine or cystine versus methionine only. Both types of strain combinations were used and both led to the successful isolation of balanced heterokaryons, as presently described.

Balanced heterokaryons between dwarf strains. The first combination attempted between two dwarf strains was that of *22y dw*1 and *7w dw 4*, i.e. of one dwarf strain with yellow conidia and requiring adenine or hypoxanthine, and another dwarf strain with white conidia and requiring cystine or thiosulphate. On MM agar, strain *7w dw 4* does not grow at all; strain *22y dw 1* grows to a barely visible size (Pl. 1, fig. 1). On CM agar *7w dw 4* forms very tiny dome-shaped colonies and *22y dw 1* irregularly shaped colonies, highly convoluted and seldom growing beyond 1 cm. diameter (Pl. 1, fig. 1).

The balanced heterokaryon was obtained by incubating for 5 days in a test-tube in liquid CM a thick mixed suspension of conidia of the two strains, removing the mycelium formed, teasing this out on the surface of MM agar plates and incubating for a further 9 days. After this time a little growth had occurred at various points, and out of some of these there grew small tufts of more vigorous mycelium with white heads. Isolation of growing tips from these tufts on to MM agar plates gave uniformly good growth with macroscopically white sporing surface (Pl. 1, fig. 1). Isolation on to CM agar gave mainly white growth of the kind just mentioned, alternating with patches obviously of *22y dw 1* type.

Table 2. *Plating on supplemented medium of conidia from balanced heterokaryons in order to recover the two component types*

		Colonies obtained			
		Yellow		White	
Balanced heterokaryon	Medium	No.	%	No.	%
22y dw 1 + 7w dw 4	CM	222	78·2	62	21·8
22y 13 + 7w 16	MM + adenine + methionine	16	8·5	171	91·5

That most of the mycelium on MM agar, and most of the whitish regions of the mycelium on CM agar, were heterokaryotic and balanced was suggested by their growth rate and non-dwarf habit, and was proved by the following tests: (1) successive transfers both on MM agar and on CM agar, of hyphal tips from heterokaryons grown on MM or on CM, gave growth with the characters just described; (2) a proportion of single hyphae isolated from heterokaryons gave origin again to heterokaryotic growth; (3) platings of conidia from colonies derived from single hyphae gave the two types of parental strains: *22y dw 1* and *7w dw 4* (Table 2).

A second combination of dwarf strains was prepared from *7w dw 5* and *38y dw 6*. The former has white conidia and requires cystine or thiosulphate; the latter has yellow conidia and requires NH_4^+ or NO_2^-. In this case, the heterokaryons were established by isolating small bits of more vigorous mycelium which arose at the region of contact between colonies of the two types grown on CM agar. These more vigorous tufts were quite evident after

12 days from the inoculation of the plates. Evidently, even on CM agar, the heterokaryon between the two dwarf strains used had a sufficient selective advantage over either dwarf component to be able to escape from the homokaryotic mycelia.

Mass hyphal transfers on to MM agar from these more vigorous tufts grew at a rate approaching that of the wild type and developed a macroscopically yellowish sporing surface. No further work was carried out with this combination.

Following the notations used in previous papers (e.g. Pontecorvo, 1953*b*), heterokaryons are designated by the code numbers of the components, joined by the sign +. Thus the two heterokaryons mentioned so far will be designated: *22y dw1* + *7w dw4* and *7w dw5* + *38y dw6*, respectively.

Balanced heterokaryons between strains each having two nutritional requirements. Balanced heterokaryons were synthesized between strains *22y13* (yellow, requiring adenine or hypoxanthine, and methionine; inhibited by cystine) and *7w16* (white, requiring methionine or cystine or thiosulphate, and adenine; inhibited by hypoxanthine). Neither of these two strains shows any growth on MM agar and their conidia do not even germinate on it. They both grow well on MM agar supplemented with the respective pairs of growth factors; *22y13* grows well and *7w16* grows poorly on CM agar.

The balanced heterokaryons *22y13* + *7w16* were obtained in the same way as in the case of *7w dw5* + 38*y dw6*, i.e. by isolation on to MM agar of tufts of vigorous mycelium arising from the region of contact between colonies of the two strains growing on CM agar. The selective advantage of the heterokaryon on CM agar may perhaps depend, in this case, on overcoming the inhibitions mentioned above.

The heterokaryon grows well on MM agar; macroscopically its sporulating surface is yellowish. The same three tests mentioned before gave proof of the heterokaryotic condition: (1) perpetuation of the heterokaryon by mass transfers of hyphae on MM agar; (2) perpetuation of the heterokaryon by single hypha isolation; (3) recovery of both component strains by plating conidia of colonies grown from single hyphae (Table 2). An additional visual test was that on addition of the appropriate growth factors to MM agar, the white, the yellow or both components would sector out.

Properties of heterokaryons. The balanced heterokaryons of *Penicillium chrysogenum*, in contrast with those of other species (*Aspergillus nidulans*, Pontecorvo, 1953*b*; *A. niger*, Pontecorvo *et al.* 1953) can be maintained by massive transfers from the sporing surface. As in those other species, the conidia of *P. chrysogenum* are uninucleate (Tonolo & Urbani, 1952). Thus in a heterokaryon/nuclei of each kind should segregate when the conidia are formed. That this is substantially so is shown by the results of platings of conidia at density of the order of 100 conidia/plate (Table 3). However, when plating massive numbers of conidia, rare heterokaryotic colonies arise within 48 hr., at the rate of a few per 10^6 plated conidia (Table 3; Pl. 1, figs. 2, 3). It is probable that these colonies originate from bits of heterokaryotic mycelium, accidentally present in the inoculum.

As mentioned before, one of the three heterokaryons formed (*22y dw 1* + *7w dw 4*) has macroscopically a white sporing surface; the other two have yellowish surfaces. The structure of the penicillus in *Penicillium chrysogenum*, with loose and divergent conidial chains, makes it impossible to tell whether chains of different colour arise from the same conidiophore. Furthermore, the two colours (white and yellow) are not sharply distinguishable under the microscope. Nevertheless, the fact remains that all heterokaryons have, in different proportions, both white and yellow penicilli but no green ones, while diploids (see below) have green penicilli. The colour of individual conidia seems thus to depend upon the kind of nucleus segregated into each of them. In this respect the colour markers used in *P. chrysogenum* behave more like those used in *Aspergillus nidulans* (Pontecorvo, 1953*b*) than like those used in *A. niger* (Pontecorvo *et al.* 1953).

Table 3. *Heterozygous diploids obtained from conidia of balanced heterokaryons plated in minimal medium*

	Plated conidia		New heterokaryotic colonies arising*		Diploids detected as†			
					Whole colonies		Sectors from heterokaryotic colonies	
Balanced heterokaryons	Total no.	No. per dish	Total	Per 10^6 plated conidia	Total	Per 10^7 plated conidia	Total	Per 10^2 colonies
22y dw 1 + *7w dw 4*	77×10^6	$2{\cdot}6\times10^6$	769	10	2	0·25	6	0·77
22y 13 + *7w 16*	$8{\cdot}4\times10^6$	$0{\cdot}6\times10^6$	1083	138	3	3·6	16	1·5

* When plating conidia of balanced heterokaryons at densities of the order of 10^6 per plate a few tens of colonies arise within 48 hr. on MM. They turn out to be balanced heterokaryons, presumably arising from bits of mycelium or from rare bi- or multinucleate conidia carrying nuclei of the two kinds. Macroscopically their sporing surface is white in the case of heterokaryon (1), or yellowish in the case of heterokaryon (2), and their morphology normal (Pl. 1, figs. 2, 3).

† Diploids are identified either as green spored colonies of normal morphology and growth rate or as green sectors out of the newly arising heterokaryons (Pl. 1, figs. 2, 3).

Isolation of heterozygous diploids

Diploid from heterokaryons 22y dw 1 + *7w dw 4.* From heterokaryons grown for 2–3 weeks on MM agar, conidia (washed twice in water) were plated on MM agar at the rate of $2{\cdot}6\times10^6$ conidia/plate. After 48 hr. a mean of about twenty-eight colonies/plate were visible. After a few days' further incubation all but two of these colonies developed a macroscopically visible whitish sporing surface; tests on about thirty of them showed that they were heterokaryons, as mentioned before (Table 3). Two, however, grew better than the others and developed green penicilli. Isolation, purification by single conidium micromanipulation, and further tests (see later) showed these two colonies to be the desired heterozygous diploids. Diploids are designated by interposing a fraction sign between the symbols of the strains associated in the heterokaryon which gave them origin:

$$22y\ dw\,1/7w\ dw\,4, \quad \text{or} \quad \frac{22y\ dw\,1}{7w\ dw\,4}.$$

On keeping the plates for 2 weeks or more, about 1 % of the heterokaryotic colonies produced one macroscopically visible green sector (Table 4; Pl. 1, fig. 2). Isolation and tests of strains from such sectors shows them also to be diploids. Incidentally, after such prolonged incubation the plates show a background growth of minute colonies (Pl. 1, fig. 2) of type *22y dw1*, presumably adapted; these minute colonies represent about 5 % of the conidia of this type present in the plated suspension (Table 4).

Table 4. *Properties of heterozygous diploid* 22y dw1/7w dw4 *compared with those of the heterokaryon, of the two marked haploid strains, and of the original haploid wild type* Q176 (*see also Pl.* 1, *fig.* 1)

Strain	Growth habit on CM	Individual conidia		Growth factor requirements of colonies arising from		Segregation through conidia and sectors
		Colour	Size	Single conidia	Hyphae	
Q176 (haploid)	Normal	Green	Normal	None	None	None
22y dw1 (haploid)	Dwarf	Yellow	Normal	Hypox.	Hypox.	None
7w dw4 (haploid)	Dwarf	White	Normal	Cystine	Cystine	None
22y dw1+7w dw4 (heterokaryon)	Normal	White or yellow	Normal	Hypox. or cystine	None	Parental types, except for very rare diploids (10^{-7})
22y dw1/7w dw4 (diploid)	Normal	Green	Giant	None	None	Small proportion (10^{-2}) of parental and new types

Diploid from heterokaryon 22y13+7w16. From heterokaryons grown for about 2 weeks on MM agar, conidia (washed twice in water) were plated on MM agar at the rate of $0{\cdot}6 \times 10^6$ conidia/plate. The results were similar to those of the previous example with the difference that, (*a*) the newly arisen heterokaryotic colonies had macroscopically yellowish sporing surface; (*b*) relative to the number of conidia plated, the proportions of newly arisen heterokaryotic colonies and of the green diploid colonies was about ten times higher (Table 4); (*c*) the green sectors from the heterokaryotic colonies were about twice as frequent; (*d*) there was no delayed background growth of parental types (Pl. 1, fig. 3). Isolation from one of the green colonies, purification by single conidium micromanipulation and further tests led to the establishment of diploid *22y13/7w16*.

Properties of diploids. The diploids differ from the heterokaryons, from the haploids which formed the heterokaryons and from the haploid wild type in a number of ways. These have been tabulated (Table 5) for the case of diploid *22y dw1/7w dw4* and some of them are evident on Pl. 1, fig. 1. These differences also apply, *mutatis mutandis*, to the case of diploid *22y13/7w16*. The size of the conidia is not easily measurable because of considerable variations in shape. However, a visual comparison (Pl. 1, fig. 4) leaves no doubt that diploids have larger conidia, a fact suggesting that Sansome (1949) was right when she deduced that the 'gigas' strains isolated by her in the closely related species *Penicillium notatum* were (homozygous) diploid. As diploids heterozygous for nutritional requirements, colours of conidia and habit of growth, show features approaching those of the wild type (no requirement, green conidia and normal growth) all the mutant properties used as markers in this work are recessive. In the diploids segregation and recombination take place, as shown in detail in the next section, i.e. a small proportion of individual conidia or hyphae of the diploids give rise to strains different from the diploid from which they arose, in that they show one or more properties of the original marked strains associated either in the original way or in new ways.

Table 5. *First-order segregants from diploid:*
22y dw 1 (*yellow, hypoxanthine-requir., dwarf-1*)
7w dw 4 (*white, cystine requir., dwarf-4*)

	Prototrophs	Requiring hypoxanthine	Requiring cystine	Requiring both	Total
		A. Obtained as whole colonies*			
Green					
Dwarf	5	0	0	0	5
Normal	964	1	0	0	965
Yellow					
Dwarf	2	1	0	0	3
Normal	2	0	0	0	2
White					
Dwarf	6	0	0	0	6
Normal	12	0	0	0	12
Non-sporing					
Normal	17	0	0	0	17
	1008	2	0	0	1010
		B. Obtained as sectors†			
Green					
Dwarf	0	0	0	0	0
Normal	0	0	1‡	0	1
Dwarf	1	0	0	0	1
Yellow					
Normal	5	0	0	0	5
White					
Dwarf	0	0	0	0	0
Normal	14	0	0	0	14
	20	0	1	0	21

* Conidia of *22y dw 1/7w dw 4* were plated on CM, and 1010 colonies were classified as to colour, morphology and requirements.

† Sectors, differing in colour or growth habit from the mother colonies, were isolated from a proportion of the 964 green, normal, prototroph colonies, and classified as to colour, morphology and requirements.

‡ Though this sector had green conidia and non-drawf habit, it was isolated because of its rather thinner growth than the rest of the mother colony: it turned out to be cystine-requiring.

Segregation and recombination in diploids

In *Aspergillus nidulans* and *A. niger* (Pontecorvo & Roper, 1953; Pontecorvo *et al.* 1953; Pontecorvo *et al.* 1954) mitotic segregation and recombination occurs once about every 100 divisions in heterozygous diploids. Its detectable result is the production of diploid nuclei homozygous for one or more of the recessive markers or of haploid nuclei with one or more of the recessive markers. A patch of mycelium homokaryotic for one of these nuclei (for short, 'segregant' nuclei) will have the recessive marker property or properties. When these properties are detectable by inspection (e.g. colour of conidia) the patch of mycelium may be identified visually and a strain isolated from it. In the two other species mentioned above this visual identification is easy even in the case of a single head in a background of thousands with the wild type colour. In *Penicillium chrysogenum* individual heads differing in colour from the background are difficult to recognize, and only patches of homokaryotic segregant mycelium with a substantial number of segregant heads can be identified visually and isolated.

Thus one way of detecting mitotic segregation and recombination of colour markers is to examine a number of diploid colonies (preferably originated from single conidia) for the presence of sectors with penicilli of one of the recessive colours; isolation from these patches will permit the classification of each segregant in respect of other properties also (e.g. nutritional requirements, growth habit, etc.). Another way in which segregation, not only of colour markers but of any markers, can be recognized is by plating conidia of the diploid and classifying individually the resulting colonies. This classification can be visual as to colour of conidia and growth habit, but requires the testing of individual colonies for nutritional requirements. Segregation and recombination was analysed extensively in the case of diploid *22y dw 1/7w dw 4* (see later). As to diploid *22y 13/7w 16*, we only went so far as to observe that it did segregate for colour markers. An extensive analysis of this and other diploids was carried out later at the Istituto Superiore di Sanitá, Rome, by one of us (G. S.), and it will be the subject of a separate publication.

First-order segregants. First-order segregants from diploid *22y dw 1/7w dw 4* were obtained by plating conidia of a subculture (purified by single conidium micromanipulation) on fully supplemented medium; 1010 colonies (Table 5) from this plating were transferred to plates of minimal agar. After 2 days those colonies which showed no further growth were picked out and tested for nutritional requirements and colour. The remaining colonies were transferred back on to supplemented medium, kept until the colour appeared on the sporing surface, and then classified for colour and morphology.

A proportion of the 964 green prototroph normal colonies (out of the total of 1010) showed sectors; some were yellow, some white and some non-sporulating or with a growth habit different from that of the mother colony. Twenty-one of these sectors were isolated, each from a different colony, and further tested. Table 5 shows the classification of the forty-six segregants obtained as whole colonies after plating, and of the twenty-one segregants detected as sectors in some of the colonies arising from platings. The proportion of segregants among the conidia of the diploid is about 5 % (46/1010). No recombination of parental *recessive* characters occurred among the sixty-seven first-order segregants isolated either as colonies or as sectors (Table 5).

Second-order segregants. The first-order segregant with green conidia, normal growth and hypoxanthine requirement was tested for further segregation after purification by single conidium isolation. One yellow and two white sectors from colonies of this strain gave yellow or white, hypoxanthine-requiring strains, with normal growth. A white hypoxanthine-requiring strain is an example of recombination between recessive properties of the starting strains.

CONCLUSIONS

The experiments reported show the occurrence in *Penicillium chrysogenum* of the parasexual processes already found in two other species of filamentous fungi. In Penicillium the analysis has gone only so far as to identify three out of the four steps which were studied in detail in the two other species, namely: (1) formation of heterokaryons; (2) formation of heterozygous diploid nuclei within heterokaryons; (3) recombination in the diploid nuclei. We have not yet looked for and identified the formation of haploid nuclei from diploid nuclei (Pontecorvo *et al.* 1953*a*; Pontecorvo, 1954). As, however, this is a consequence of accidents of mitosis of a type known to occur in all higher and lower organisms in which it has been looked for, it is a reasonable assumption that it will be found also in Penicillium.

Penicillium thus may have, like the two other species, a complete parasexual cycle (Pontecorvo, 1953*a*). This involves alternation of haploid and diploid stages, the possibility of storing gene variation under the cloak of dominance in both heterokaryons and heterozygotes and the possibility of gene recombination.

Because of its low growth rate and certain details of its morphology, *Penicillium chrysogenum* is a species much less suitable than *Aspergillus nidulans* or *A. niger* for the detailed analysis of the parasexual processes themselves. It is, however, a species of great economic importance. The discovery of the parasexual processes in it is relevant in two respects. First, this third species of filamentous fungus again shows the occurrence of these parasexual processes; thus they would appear to be widespread at least. Secondly, the parasexual processes in *P. chrysogenum* can be used for practical purposes: (*a*) deliberate 'cross-breeding' for the production of improved industrial strains; (*b*) the identification of different genetic blocks in the analysis of the biosynthesis of penicillin.

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EXPLANATION OF PLATE

Fig. 1. +, the haploid green wild type; 1, the haploid dwarf, yellow, hypoxanthine-requiring *22y dw 1*; 4, the haploid dwarf, white, cystine-requiring *7w dw 4*; HK, the heterokaryon between *22y dw 1* and *7w dw 4*; D, the diploid derived from the heterokaryon. Left: on complete medium. Right: on minimal medium. White circles indicate the positions of inoculum on MM of the two dwarf strains.

Fig. 2. Plating on MM of conidia of heterokaryon *22y dw 1* + *7w dw 4* ($2{\cdot}6 \times 10^6$/dish): there are about twenty heterokaryotic colonies (large) per dish, and a background of minute colonies of type *22y dw 1*. About 1 % of the heterokaryotic colonies show, like the one in the centre, a green diploid sector.

Fig. 3. Plating on MM of conidia of heterokaryon *22y 13* + *7w 16*: results similar to those of Fig. 2, but no background growth. Two heterokaryotic colonies show green diploid sector.

Fig. 4. Conidia of heterokaryon *22y dw 1* + *7w dw 4* (left) and of the diploid derived from it (right) about ×400.

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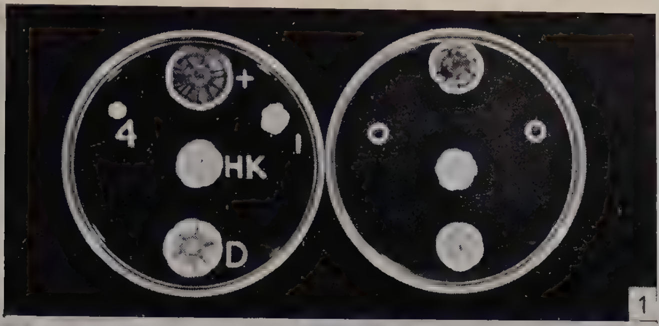

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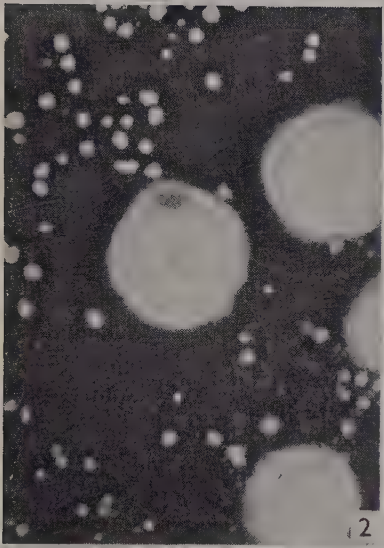

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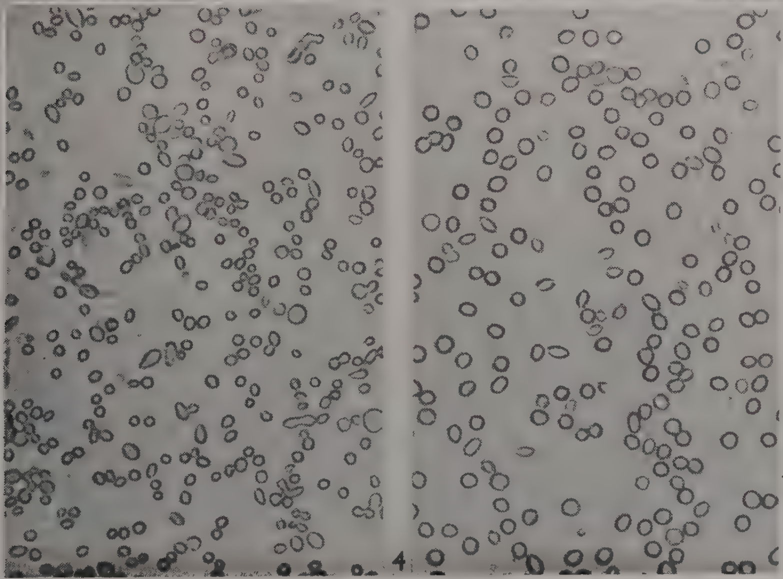

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G. Pontecorvo & G. Sermonti—Parasexual recombination

DICKINSON, L. (1954). *J. gen. Microbiol.* 11, 105–113.

The Behaviour of a Temperate Phage of *Pseudomonas aeruginosa* compared with that of a Serologically related, Virulent Mutant

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SUMMARY: A temperate phage of *Pseudomonas aeruginosa* (*P. pyocyanea*), on addition to a non-lysogenic indicator strain of the organism, produced a lysogenic culture with a high spontaneous rate of free phage production. A virulent mutant, serologically related to the temperate phage, was obtained from this lysogenic culture; this mutant did not give rise to lysogenically infected bacteria. Both virulent and temperate phages, acting on an indicator strain, had latent periods of 90 min., and the multiplication of each was inhibited by a phenanthridine added immediately after adsorption. This indicated that the initial stage of infection is the same for both types of phage. The production of free phage (i.e. the prophage to phage change) by the lysogenic culture derived from the temperate phage was not inhibited by the drug.

Filtrates of lysogenic strain C7X of *Pseudomonas aeruginosa* yielded two types of plaques when assayed against an indicator strain (Dickinson & Codd, 1952). One type was clearly visible after 18 hr. incubation of the assay plate while the other gave hazy plaques, only visible after 48 hr. In view of Boyd's (1951) work on lytic and symbiotic phages of *Salmonella typhimurium*, the results of further investigations into the case of *P. aeruginosa* are of interest. Lwoff & Gutman (1950) and Lwoff, Siminovitch & Kjeldgaard (1950), using *Bacillus megaterium*, considered that whether or not phage was reduced to prophage in a given bacterium was primarily a function of host resistance. Lieb (1953) analysed the factors involved in the establishment of lysogenesis in a strain of *Escherichia coli* and suggested that the cell response was due to physiological conditions. Lwoff (1953) has recently reviewed the subject of lysogeny. The present work compares the behaviour of a temperate phage with a virulent mutant.

Definitions. In this paper the terms proposed by Jacob, Lwoff, Siminovitch & Wollman (1953) are used, in particular:

Temperate phage, meaning a phage capable of producing a lysogenic system with a given host.

Virulent phage, meaning a phage incapable of producing a lysogenic system with a given host.

Cultures are denoted by C and a culture, e.g. C10, made resistant to a given phage, Pc, is denoted by C10/c. The *total* phage count of a culture means the number of infectious centres, including free phage. The *free* phage count is that obtained when all bacteria have been removed.

METHODS

Strains. Iridescent strains C1 and C7X and the non-iridescent indicator strain C10 (Dickinson & Codd, 1952) were employed. The phages produced by these strains are noted in Table 1. Phages Pa and Pb, originally isolated from C1 (Dickinson, 1948), had been passaged on C10 at intervals, using a phage:bacteria ratio of about 1:1000. These phages were quite distinct serologically and were used only for typing strains.

Phage preparations. Except where otherwise stated, phage preparations were obtained by filtration through Ford Sterimats. Several recent batches of Sterimats adsorbed nearly all the phage; through the courtesy of Mr D. McLean Wyllie of Messrs T. B. Ford Ltd., samples of the raw materials of the mats were examined. As a result of this work Messrs Ford supplied a new grade of mat, SB/B, which was satisfactory for phage filtrations.

Titration of phages. Phage dilutions (1 ml.) in Ringer's solution were added to 1 ml. indicator and 4 ml. nutrient agar and the mixture was poured on to nutrient agar plates. Assay plates were read after 18 hr. incubation at 37°.

Determination of latent periods. The method of Dickinson & Codd (1952) was used.

Cross-resistance tests. Cultures were spread on nutrient agar plates and dried in the incubator. Undiluted filtrates from the cultures under test were spotted on this; when required, resistant organisms were recovered from the spotted area.

Serological tests. Phage antisera were prepared in rabbits inoculated intravenously with 1 ml. high titre filtrates once weekly for 6 weeks. All sera were inactivated by heating at 56° for 20 min. and then kept at −20°.

For preliminary neutralization tests equal volumes of sera dilutions were mixed with dilutions of the phage preparation, and the mixture incubated at 37° for 30 min. in a water-bath.

For determining the rates of neutralization of phages, 25 ml. of the phage preparation and 0·5 ml. antiserum were incubated at 37° and samples removed for phage assay at the intervals 3, 15, 45, 75 min., 3, 5 hr. Controls, using normal serum, showed no loss in titre at 5 hr.

EXPERIMENTAL

Derivation of temperate (Pz) and virulent (Pc) phages

The derivation of the virulent and temperate phages appears in Table 1. There were essentially three types of phage plaques in this work: (1) uniformly hazy; (2) those with a solid centre surrounded by a zone of complete lysis; (3) uniformly clear. A hazy plaque from a 48 hr. assay plate of a filtrate of C7X on C10 was passaged twice on C10, using single plaque isolations. The resulting culture, C10/h, was lysogenic and of twenty colonies tested all produced phage, giving a mixture of hazy plaques with about 1 % of the zoned type; after passage on C10 even the hazy plaques were visible at 24 hr. but they never showed a lytic zone. These hazy plaques were often extremely difficult to count.

Unlike C7X, C10/h was not iridescent but, like C7X, it was resistant to both typing phages Pa and Pb. It was difficult to prove whether all the zoned plaques present were the result of phage mutation or whether different phages were involved. All hazy plaques were neutralized by antiserum to Pb but some of the zoned plaques were neutralized by antiserum to Pa, whereas others were neutralized by antiserum to Pb. One zoned plaque, which gave progeny neutralized by antiserum to Pb, was selected for further passage on C10.

Table 1. *Derivation of phages of* P. aeruginosa

		Filtrate of culture			Culture obtained by phage passage on C10		
Source	Ref. no.	Phages isolated	Plaque description after C10 passage	Serological type of phage (Pa or Pb)	Lysogenic	Resistant to Pa	Resistant to Pb
War-wound	C1*	Pa	Zoned	Pa	+	+	−
		Pb	Zoned	Pb	+	−	+
Lab. isolate	C7	.	.	.	.	−	−
C7+C1 filtrate (mouse passage)	C7X*	P18	Zoned	Pa	?	+	−
		Ph	Hazy	Pb	+	+	+
Lab. isolate	C10	.	.	.	.	−	−
C10+Ph	C10/h	Ph	Hazy	Pb	+	+	+
Single colony of C10/h	C10/h	Pz	Zoned	Pb	+	−	+
C10+Pz	C10/z	Pz	Zoned	Pb	+	−	+
		Pc (rare)	Clear	Pb	−	−	+
C10+Pc	C10/c	Pc	Clear	Pb	−	−	+

* = iridescent culture; ? = not studied in detail.

Three consecutive single-plaque isolations of this zoned plaque from a filtrate of C10/h were made, the resulting culture being termed C10/z. For two passages, only zoned type plaques were observed. After the third passage the titre of the filtrate was 37×10^8/ml. but at the $1/10^6$ dilution there were five absolutely clear plaques. One of these was picked off with the C10, incubated and filtered (free phage titre, 10^9/ml.); all the plaques were of the same clear type and this phage was termed Pc in contrast to Pz (zoned type plaque) and Ph (hazy type plaque).

Relationship between phages Pb, Ph, Pz and Pc

Preliminary neutralization tests, using a 30 min. contact period at 37°, indicated a serological relationship between Pb, Ph, Pz and Pc. Antiserum to Pa had no effect on any of these phages, less than ten phage particles being neutralized by 1 ml. serum even after 3 hr.; 1 ml. antisera to either Pb or Pc neutralized more than 10,000 phage particles of all four phages, but antiserum to Ph was much less active against Pb and Pc than against Pz and Ph.

The rates of neutralization by two sera against the four phages are shown graphically in Fig. 1. Antiserum to Pc produced a very rapid decrease in titre; as soon as readings were practicable (about 3 min.) this was apparent and at

15 min. all counts were zero. Antiserum to Pb gave a similar result, all counts being zero at 20 min.

Antiserum to Ph gave less steep slopes against all four phages, but particularly was this evident against Pc and Pb. The Pb curve levelled out after 3 hr. but the initial slope was almost exactly equal to that of Pc; this slow rate of inactivation explains the apparent lack of action on Pb and Pc by this antiserum in the 30 min. test. The slopes of the Pz and Ph curves are steeper than those against Pb and Pc but the rate of inactivation is less than that produced by antisera to Pc. The two antisera prepared against Pz were not very potent. A 1/25 dilution was inactive against even the homologous antigen but a 1/10 dilution gave slopes similar to those produced by antiserum to Ph.

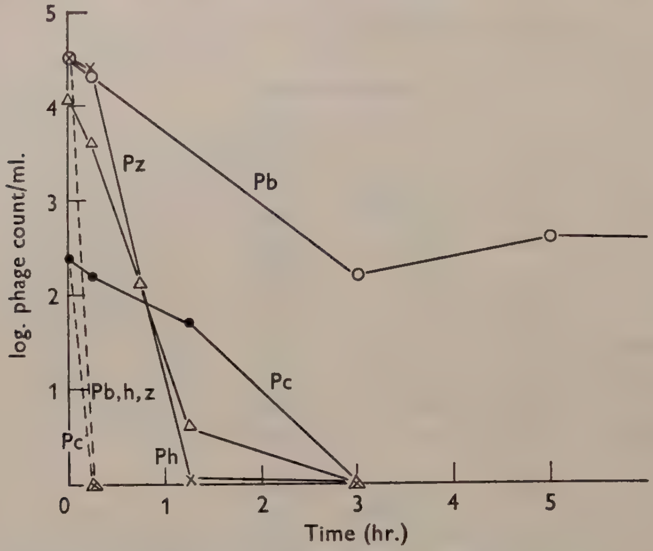

Fig. 1. Rates of neutralization of phages Pb (○—○), Ph (△—△), Pz (×—×) and Pc (●—●) by antisera to Ph (unbroken line) and Pc (broken line). The lines for Pb, Ph and Pz against antiserum to Pc are so close that they appear as one in the diagram. Controls with normal serum showed no loss in titre at 5 hr.

It appeared that Pz and Ph were identical except for the appearance of plaques formed. On certain batches of medium, distinguished by the fact that on them C10 produced a dirty blue-green pigment instead of the normal yellowish green, Pz preparations gave a mixture of zoned and hazy plaques, whereas the same preparation gave only zoned plaques on the normal agar. The only difference traced to the medium was the method of digestion of the meat; the normal batches were tryptic or weak papain digests, the abnormal were concentrated papain digests.

Cross-resistance tests showed no difference between any of these phages. Cultures made resistant to any one were resistant to all, but still sensitive to Pa.

Comparison of culture of C10 treated with phages Pz and Pc

Three well-isolated plaques of each type of phage, Pz and Pc, were picked off into broth (plus accompanying C10) and the cultures, C10+Pz and

C10+Pc, examined after incubation. The following observations were made: (1) the total phage count of the unfiltered culture, and the free phage count of the filtrate were found and all plaques observed; (2) the cultures were plated out for individual colonies, followed by subculture into broth (for phage production) and to agar (for resistance tests); (3) cultures derived from single colonies of C10/z and C10/c were grown in the presence of compound B (2:7-bis-(-2-dihydroglyoxalinyl)-9-phenylphenanthridine trihydrochloride). This compound was known to prevent the reproduction of phage Pb on C10, when added within a few minutes of infection, but to have little effect on the total phage titres of lysogenic cultures (Dickinson & Codd, 1952; Mills, 1953). The cultures were diluted $1/10^4$ in broth containing 1 mg. compound B/ml. Three passages were made in this medium and the cultures were then titrated for total phage and tested for their ability to produce phage in the absence of the drug.

Total and free phage counts were about 10^8–10^9/ml. for both C10+Pz and C10+Pc cultures. Examination of the assay plates, involving about 10,000 plaques, revealed *only* Pc type from C10+Pc cultures and filtrates. There was only one clear plaque amongst the thousands of the Pz plaques examined from C10+Pz. All broth cultures derived from Pc or Pz plaques were resistant to Pz, Pc and Pb phages but not to Pa.

The thirty single colony cultures tested from C10+Pz were resistant to Pz, Pc and Pb phages but sensitive to Pa; all these thirty broth cultures produced phage. It was difficult to obtain colonies from C10+Pc cultures (containing only few unlysed organisms) except where free phage was carried over. On second subculture, however, phage-resistant but *not* phage-producing colonies were obtained. Compound B had little effect on the phage titres of cultures of C10/z; both treated and untreated cultures gave high titres even after three passages. C10/c cultures did not produce phage.

These results showed that the serologically related phages Pz and Pc established different relations with C10. Pz set up a lysogenic system; with Pc no lysogenic organisms were obtained. Pl. 1, figs. 1 and 2, show the plaques produced by Pz and Pc.

Properties of temperate phage Pz and virulent phage Pc: stability. Preparations of Pz and Pc in broth or Ringer's solution maintained their titres when held at 55° for 30 min., 37° for 4 days and at 18 and 4° for 6 months. Dilutions in 0·1 M-phosphate citrate buffers of pH's 5·0, 7·0 and 9·0 were unaffected in titres after 24 hr. at 37°. As mentioned above, Pz preparations on some media gave mixtures of hazy and zoned plaques.

Latent period. There was an increase in phage titres at 90 min. for both Pz and Pc when acting on C10. Each tube contained about 10–50 phage particles and 1000 host cells; the average phage titres rose from 14 to 71 in the case of Pz and from 43 to 113 for Pc. When a culture of C10/z was diluted so that each tube had both a host count and total phage count of about 10/tube there was no regular time for a rise to occur in all tubes. Taking readings up to 5½ hr., one tube showed an increase in titre (forty plaques) at 3½ hr., but six others remained at the initial level up to 5½ hr. With an average initial level of one bacterium/tube none of the ten tubes showed an increase of phage titre within $5\frac{1}{2}$ hr. Four of these presumably contained no bacteria; after $5\frac{1}{2}$ hr. incubation half of the contents of each tube was plated out for bacterial colonies and the other half assayed for phage, but even on prolonged incubation (48 hr.) of the respective assay plates there was neither colony nor plaque. The above results indicated that only a proportion of C10/z cells produced free phage (certainly less than 1 in 10, probably more than 1 in 100).

Amount of free phage in cultures of C10/z and C10/c. A broth culture of C10/z (colony count 10^8/ml.) had a total phage count of $1{\cdot}27 \times 10^8$/ml.; after heating at 55° for 30 min. to kill the bacteria, the free phage count was $4{\cdot}7 \times 10^7$/ml. Bacteria-free filtrates and cultures treated with 50 % (v/v) glycerol for 13 days at room temperature also had high free phage titres, never less than one-tenth of the culture titres. Similar results were obtained for lactate medium except that filtrates (Sterimats and Gradocol membranes) gave negative results; this is usual for the phages of *P. aeruginosa* in lactate medium of Ringer's solution, since marked adsorption occurs.

When phage Pc was grown on C10 in broth or lactate medium the resulting cultures contained up to 10^{10} total phage particles/ml. and there was no decrease in titre when the bacteria were killed. Broth filtrates, but not lactate medium filtrates, also had very high phage counts.

Effect of varying the phage : host ratios for phages Pz and Pc. To see whether the infection ratio of the phages affected the culture response, each phage was serially diluted and the tenfold dilutions added to serial dilutions of C10 in broth. After incubation the cultures were spotted on to C10. All Pc tubes yielded clear lytic spots and all Pz tubes gave centred spots. Ten colonies from each culture containing the phage : host ratios $10^8:10^8$, $10^8:10$, $10:10^8$ and 10:10 were examined for phage production and resistance to Pc and Pz. All forty colonies from C10/z tubes were phage-producing and resistant. From C10/c tubes, mixtures of resistant-non-phage producing organisms and unchanged C10 were obtained; out of 100 resistant colonies none produced phage. Thus, multiple infection by Pc did not establish lysogenesis.

Growth of mixtures of phages Pz and Pc on C10. As expected, the result of adding the mixed phages, in Pz:Pc proportions of $10^6:10^6$, $10^6:10^2$, $10^2:10^6$ and $10^2:10^2$, was the rapid elimination of Pc; even where Pc was present in 10,000-fold excess it was eliminated within four subcultures.

Action of compound B on growth of phages Pz and Pc

One ml. of a 24 hr. broth culture of C10 and 1 ml. of the phage filtrate, diluted to contain about 10^5 phage particles/ml., were added to 100 ml. broth. After a 5 min. adsorption period, 1 ml. of the mixture was added to fivefold dilutions of compound B in broth. After incubation for 18 hr. the tubes were tested for the presence of phage by spotting on to C10. Results, expressed as:

$$\frac{\text{amount drug (mg./ml.) inhibiting phage growth}}{\text{amount drug (mg./ml.) inhibiting host growth}}$$

were the same for Pz and Pc, i.e. $0{\cdot}016/>0{\cdot}4$. Total phage counts for both

Pz and Pc controls were 10^8 to 10^9/ml., while total phage counts for the treated cultures remained at the initial level. Even when cultures were incubated for 7 days the phage did not increase in the treated cultures; however, such treated cultures still produced phage to high titre when subcultured into drug-free medium. The activity of the compound against Pz acting on C10 was in contrast to its lack of action on the phage production in established lysogenic cultures of C10/z.

Before seeking an explanation for this behaviour, evidence that the effect of the phenanthridine was not due to action on free phage or on the adsorption of phage on to host was needed. Both Dickinson & Codd (1952) and Mills (1953) showed that the action of this compound on Pb phage could not be accounted for by the slight viricidal action at 37°. Mills also found that when adsorption was carried out in the presence of the drug and the phage (Pb) host (C10) suspension, then diluted 1/1000 before distribution into the latent-period test tubes (i.e. to below the effective concentration of the drug), then a burst at the normal time still occurred. When adsorption was carried out in the control medium, the drug then prevented phage growth if added within 3 min. of the dilution of the adsorption mixture.

These facts were confirmed for phages Pz and Pc in latent-period experiments. The phenanthridine prevented phage growth when added immediately after the dilution of the adsorption mixture, but had no effect when the drug addition was made at 15 min.

Total and free phage titres of phage cultures C10+Pz, C10+Pc and C10/z with and without compound B. In all the following experiments compound B was used at 0·4 mg./ml. in nutrient broth. Total phage was assayed in the usual way and free phage was estimated after heating the cultures (diluted 1/100) at 55° for 30 min. The dilution was necessary because at 55° compound B had a marked viricidal action at 0·4 mg./ml. on both Pz and Pc; the bacteria in the diluted culture were all killed after 30 min. It should be noted that in all control cultures the free phage counts are almost as high as the total phage counts (Table 2). In all cases of C10+Pz and C10/z the total phage counts were probably low since not every lysogenically infected bacterium will produce a plaque; the error cannot be very great since both the free phage titres and the host colony counts were of the order of 10^8/ml. Since there were only resistant (non-lysogenic) bacteria in 24 hr. cultures of C10+Pc, the total and free phage titres were the same.

Table 2 shows that compound B suppressed the rise in titres of Pz and Pc when acting on C10; incidentally, it confirmed the fact that there was no contact action. The important point is that it had no effect on the free phage titre of C10/z, i.e. it did not prevent the development of prophage to phage in this culture.

DISCUSSION

C10/z, produced by the action of the temperate phage Pz on C10, is a typical lysogenic culture. As high phage titres are obtained in lactate medium it does not appear that any external amino acids are essential for phage maturation, unlike the temperate phage of *Escherichia coli* (Gots & Hunt, 1953). However, the finding of Gots & Hunt referred to an inducible system, whereas C10/z has a high spontaneous rate of phage production.

Although phage Pc arose as a mutant of phage Pz it is not responsible for the lysis of the cells in C10/z culture, for if it were one would expect to find frequently the clear plaques of phage Pc. In fact, in the latent period experiments using ten cells of a C10/z culture, all the plaques which were produced at $3\frac{1}{2}$ hr. were zoned. Only a proportion of the cells (between 1:10 and 1:100) produced phage spontaneously, as in other lysogenic systems. Phage Pc is a typical virulent phage and no lysogenically infected organisms have been obtained from it, only resistant non-phage producing bacteria. It is soon eliminated when in competition with Pz for the same host, because it cannot attack either the lysogenically infected organisms, produced by the action of Pz, or the C10/c organisms.

Table 2. *Total and free phage counts in cultures derived from temperate phage, Pz, and virulent phage, Pc, grown on C*10 *in the presence and absence of compound B. These are compared to the lysogenic culture C*10/*z*

	Counts (particles/ml.)		
	C10+Pc	C10+Pz	C10/z
Initial total phage	1×10^{5}	1×10^{5}	1×10^{4}
Control			
24 hr. total phage	5×10^{8}	$1{\cdot}3 \times 10^{9}$	$1{\cdot}3 \times 10^{8}$
24 hr. free phage	6×10^{8}	8×10^{8}	$6{\cdot}3 \times 10^{7}$
Compound B			
24 hr. total phage	$3{\cdot}6 \times 10^{5}$	$2{\cdot}8 \times 10^{5}$	$5{\cdot}8 \times 10^{7}$
24 hr. free phage	$1{\cdot}3 \times 10^{5}$	9×10^{4}	$3{\cdot}6 \times 10^{7}$

The inhibition of the action of Pz and of Pc on C10 by the phenanthridinium compound, provided that the drug is given very soon after adsorption, indicates that after adsorption the initial stages in the action of temperate and virulent phages are the same. Since the free phage titre of C10/z cultures is still high in drug-treated cultures, the change from prophage to phage cannot be affected by the phenanthridine. This suggests that the step affected may be the reduction of phage to prophage.

The relationship between the various phages Pb, Ph, Pz and Pc suggests that Pc is a virulent mutant of the original Pb, being derived as shown:

Pb → Ph → Pz → Pc.

Phages Ph and Pz are very similar except in plaque formation; Pz was derived from Ph and on certain media Pz showed many hazy plaques. Pz is not, however, identical with Pb since antiserum to Ph neutralizes Pb and Pz at different rates.

This paper is No. 3 in a series entitled 'Bacteriophages of *Pseudomonas pyocyanea*'. (*P. aeruginosa*). The author wishes to thank Mr C. E. Coulthard for his continued interest in this work, Miss M. A. Wheater for technical assistance and Mr. G. Whiting for the photograph.

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EXPLANATION OF PLATE

Fig. 1. Plaques produced by the temperate phage, Pz, of *P. aeruginosa* (×1).

Fig. 2. Plaques produced by the virulent phage, Pc, of *P. aeruginosa* (×1).

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The Effect of Oxygen on the Growth and Metabolism of the Aerobic Thermophilic Actinomycete *Micromonospora vulgaris*

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SUMMARY: In the presence of pure oxygen at one atmosphere pressure *Micromonospora vulgaris* is unable to form in liquid media a surface pellicle of growth-bearing aerial mycelium. The production of bottom growth is either unaffected or stimulated by oxygen. Vegetative mycelium which develops from spores in Warburg vessels has an oxygen uptake that is similar in the presence of air or oxygen. Aerial mycelium, harvested from growths in air, shows marked differences as regards oxygen uptake in the presence of air or oxygen; oxygen is inhibitory. Some evidence is presented to show that the effect of oxygen may be connected with the inactivation of essential —SH groups of thiol enzymes.

Full and detailed accounts of the isolation, cultivation, temperature/growth relationships and reproduction of the thermophilic actinomycete *Micromonospora vulgaris* were given by Erikson (1952, 1953*a*, *b*). She showed that this organism possesses the most complex reproductive pattern of any actinomycete yet studied, and considers that 'the device of producing a secondary aerial mycelium capable both of further growth and of producing spores, more than doubles the expected life of a mycelial organism at elevated temperatures'. Erikson & Webley (1953), in a study of the respiration of the different growth phases of this structurally complex organism, found that a high rate of oxygen uptake at 60° was associated with the presence of aerial mycelium harvested from 1- to 2-day cultures grown on liquid medium at 60°, while bottom growth harvested under similar conditions was inactive in this respect. It has now been possible to show that pure oxygen exerts an inhibitory effect on the production of aerial mycelium (in growth experiments) and metabolism of aerial mycelium (respiration experiments; Webley, 1953) of this aerobic organism. The production of vegetative growth is unaffected by these differences in the gas phase.

METHODS

Organism. The experiments presented here were performed with strain H of *Micromonospora vulgaris* (Erikson, 1953*a*).

Technique of growth. Material from 2-day-old growth on liquid CPS medium was used throughout (see Erikson & Webley, 1953).

Preparation of spore suspensions and aerial mycelium for the Warburg experiments. Spore suspensions were obtained by shaking the pellicle of growth finally obtained, after washing free of medium, in 5 ml. of M/75 phosphate buffer (pH 7·0) on a 'Microid' shaker for 5 min. This process resulted in the detachment of a high proportion of the spores which could be separated from the aerial mycelium by filtration through sterile glass wool. The spores were washed in a centrifuge and finally suspended in 2–3 ml. buffer. The resulting suspension was uniform and could be pipetted into the Warburg vessels in a similar manner to bacterial suspensions. The material remaining on the glass wool, which consisted mainly of aerial mycelium, was used for experiments with aerial mycelium. In the latter case, instead of dry-weight determinations being carried out at the end of the experiment as in previous work (Erikson & Webley, 1953), it was found less tedious to use wet weight as a means of estimation of the amount of material in the Warburg vessels. For this purpose, the sterilized Warburg vessels were first dried and weighed. The aerial mycelium remaining on the glass wool, after the preparation of spore suspensions, was next transferred aseptically into the tared vessel (care being taken to avoid the inclusion of any visible pieces of glass wool). Finally, buffer, substrates, etc., were added and the vessels attached to the manometers. Full details of running the Warburg at 60° were given previously (Erikson & Webley, 1953). For the replacement of air by oxygen the technique described by Umbreit, Burris & Stauffer (1951) was used. The manometers were first taken out of the bath and the vessels allowed to cool to room temperature before evacuation.

For convenience in this paper the final values for oxygen uptake in Figs. 2–4 are expressed as μl. oxygen uptake/100 mg. wet weight material and, in the case of Tables 1, 2 and 3 as μl. oxygen uptake/100 mg. wet weight/30 min. interval (i.e. rate of oxygen uptake during each interval). In consequence of the inherent variability of an organism of such complexity of structure it was found that in spite of all precautions not all the vessels gave uniformity in the amounts of oxygen uptake under similar conditions. Various attempts were made to overcome these difficulties, including: (1) repetition of experiments; (2) tipping substances in from the side arm or adding gases only after it was seen that the rate of oxygen uptake in the experimental vessels was initially the same as the control; (3) proportional comparison of rate of oxygen uptake before and after tipping in substances or gassing of the material in the vessels.

These details were adhered to throughout the work reported here.

Growth experiments in various gas mixtures. For this purpose, either 250 ml. filter flasks or 20 ml. filter test-tubes were employed. In the case of the flasks, 20 ml. medium was usually used while 5 ml. was sufficient for the tubes. The flasks and tubes were sterilized with a cotton-wool plug in the side tubes and necks. They were then inoculated with one or two drops of spore suspension prepared from 2-day surface growth. The cotton-wool plugs were replaced by tight-fitting sterile rubber bungs, while over the side arms were placed short pieces of pressure tubing carrying screw clips (the cotton-wool plugs being left in the side arms). The various attachments for the tubes and flasks were sterilized separately. For filling with oxygen or oxygen + nitrogen mixtures the technique described by Umbreit *et al.* (1951) for Warburg manometers, was used. Each flask or tube was alternately evacuated and filled through the side arms with the gas 3 times before the screw clip was finally closed and the flasks and tubes placed in the 60° incubator. The flasks were examined daily for surface and bottom growth.

RESULTS

Effect of pure oxygen at one atmosphere on the respiration of M. vulgaris

Vegetative mycelium developing from spores in Warburg vessels. Vegetative mycelium harvested from older cultures has previously been shown to have no measurable O_2 uptake, whereas young vegetative mycelium developing within the Warburg vessels from a suspension of developing spores is active in this respect (Erikson & Webley, 1953). Fig. 1 shows the early stages of development, as measured by O_2 uptake, of a spore suspension growing in the presence of air or oxygen. It will be seen that oxygen exerted an accelerating effect on the rate of oxygen uptake of the developing mycelium (see also growth experiments).

Table 1. *Effect of pure oxygen* (1 *atm. pressure*) *on the respiration of the aerial mycelium of* M. vulgaris *after mechanical removal of spores*

Surface growth remaining on glass wool after removal of spores in previous experiment. 50 mg. wet weight weighed into sterile Warburg vessel. Further additions 0·5 ml. M/75 phosphate (pH 7·0) + 2·0 ml. CPS medium. After 90 min. in air at 60°, air replaced by pure oxygen at 1 atm. pressure.

Time interval (min.)	μl. O_2 uptake/100 mg. wet weight mycelium during interval
	In air
0–30	52·2
30–60	142·2
60–90	272·6
90–120	Air replaced by oxygen
120–150	154·8
150–180	82·8
180–210	50·2
210–240	32·2

Aerial mycelium. When aerial mycelium remaining after the preparation of the spore suspension in the above experiment was tested in air and in oxygen, a marked inhibitory effect by the oxygen was observed (Table 1). That this inhibition was not due to exhaustion of substrate is seen from Fig. 2, where the oxygen uptake in air has been compared with that in oxygen. In this experiment, the rate of oxygen uptake (266 and 280 μl. oxygen/100 mg. wet weight/hr.) in air before replacement of air by oxygen, shows only a difference of about 6 % between the vessels as compared with the final difference after the replacement of air by oxygen (Fig. 2). The inhibition increases with time (Table 1 and Fig. 2) and is irreversible. Thus on transferring back to air after exposure to oxygen the oxygen uptake of the aerial mycelium does not rise again (Table 2).

Dickens (1946*a*) observed a similar effect of oxygen at high pressures on the metabolism of brain tissue; this effect was also irreversible. Dickens was, however, able to protect the tissue by the previous addition of certain divalent metals. These metals, in order of efficiency, were Mn > Co > Mg > Ca. Table 3 gives the results of a similar experiment carried out with the aerial growth of *Micromonospora vulgaris.* There was little evidence of a protective action on the part of these divalent metals against oxygen inhibition in the present case.

To summarize from the above experiments, the following points appear to have been established: (1) Pure oxygen (1 atm.) did not inhibit the oxygen

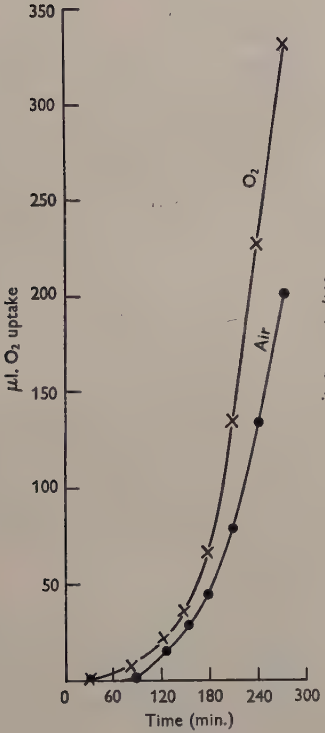

Fig. 1

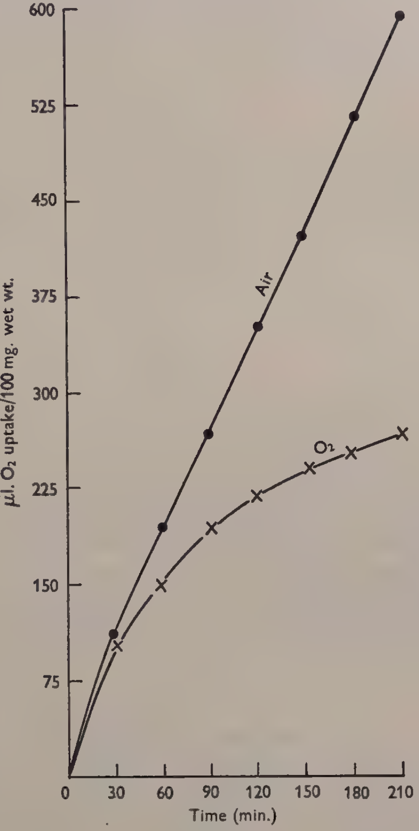

Fig. 2

Fig. 1. Effect of pure oxygen (1 atm. pressure) on the respiration of vegetative mycelium developing in Warburg vessels. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60°, washed once with M/75 phosphate buffer (pH 7·0). Shaken for 5 min. in buffer on Microid shaker subsequently filtered through sterile glass wool. Filtrate centrifuged and deposit of spores resuspended in 2 ml. M/75 phosphate buffer (pH 7·0); 0·5 ml. of this suspension placed in each vessel + 2 ml. CPS medium. Gas phase: air or oxygen, as shown.

Fig. 2. Effect of pure oxygen (1 atm. pressure) on the respiration of the aerial mycelium of *M. vulgaris. M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with M/75 phosphate buffer (pH 7·0), shaken for 5 min. in buffer on Microid shaker subsequently filtered through sterile glass wool; 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg vessels. Further additions 0·5 ml.-M/15 phosphate buffer (pH 7·0) + 1·5 ml. CPS + distilled water to give total vol. 2·5 ml. Gas phase: air or oxygen, as shown.

uptake of vegetative mycelium of *Micromonospora vulgaris* developing from spores in Warburg vessels. On the contrary, oxygen exerted a stimulating

Table 2. *Effect of replacing pure oxygen (1 atm. pressure) by air on the respiration of the aerial mycelium of* M. vulgaris

M. vulgaris (strain H); grown in liquid CPS medium for 2 days at 60° washed once with M/75-phosphate buffer (pH 7·0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into sterile Warburg vessel. Further additions 0·5 ml. phosphate (pH 7·0) + 2·0 ml. CPS medium. After 150 min. in pure oxygen (1 atm. pressure) at 60°, oxygen replaced by air.

Time interval (min.)	μl. O_2 uptake/100 mg. wet weight mycelium during interval
	In oxygen
0–30	87·0
30–60	74·6
60–90	45·3
90–120	40·0
120–150	28·0
150–180	Oxygen replaced by air
180–210	21·0
210–240	21·0
240–270	16·0

Table 3. *Effect of the presence of certain divalent metals on the inhibition of the respiration of* M. vulgaris *by oxygen*

M. vulgaris (strain H); growth in liquid CPS medium for 2 days at 60° washed once with M/75-phosphate buffer (pH 7·0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into sterile Warburg vessels. Further additions 0·5 ml. M/15-phosphate buffer (pH 7·0) + 1·5 ml. CPS medium + 0·5 ml. 0·001 M-$MnSO_4$, or 0·001 M-$CaCl_2$ or, 0·001 M-Co acetate or distilled water. After 90 min. incubation in bath at 60° vessels taken off and air replaced by oxygen except in case of control.

	Added solutions				
			0·0002 M		
Period of observation from start (min.)	Distilled water	Distilled water	$MnSO_4$	Co acetate	$CaCl_2$
	O_2 uptake by mycelium during corresponding time intervals μl. O_2/100 mg. wet wt. mycelium				
	In air				
0–30	164·4	107·6	155·7	115·6	104·3
30–60	220·8	186·5	242·8	75·2	192·7
60–90	188·4	212·8	235·1	208·5	191·5
	Vessels taken off bath; air replaced by oxygen except control				
	Air	O_2	O_2	O_2	O_2
180–210	97·2	45·9	67·2	66·7	66·8
210–240	102·0	22·4	30·4	30·7	32·9
240–270	—	—	—	—	—
270–300	124·8	22·3	31·7	27·2	20·4

effect on the rate of oxygen uptake. (2) Pure oxygen (1 atm.) had a marked inhibitory effect on respiration of aerial mycelium of *M. vulgaris*. Certain divalent metals did not protect the material against this effect of oxygen.

Growth of M. vulgaris *in air and oxygen*

Confirmation of the inhibitory action of pure oxygen upon aerial growth can be gained in a spectacular fashion, simply by growing the organism in air or in oxygen in the manner described above in Methods. In pure oxygen there is a very marked suppression of aerial mycelium while bottom growth is stimulated by the presence of oxygen. A gas mixture of 20% (v/v) O_2 + 80% (v/v) N_2, i.e. an artificial air without carbon dioxide, prepared by the methods of Umbreit *et al.* (1951) gave exactly the same type of growth as normal air; thus the effect of pure O_2 was not connected with the absence of CO_2.

Relationship of oxygen inhibition with inactivation of —SH *enzymes*

As a result of his work on the effect of high oxygen pressures on brain metabolism, Dickens (1946*b*) found that many of the oxygen-sensitive enzymes were those in which —SH groups were known to be present. With this in mind, the action of various —SH enzyme inhibitors was tested on the respiration of the aerial mycelium of *Micromonospora vulgaris.* From Fig. 3 it will be seen that 0·001M-sodium iodoacetate markedly inhibited the oxygen uptake of the aerial mycelium. On the other hand, 0·001M-sodium azide (an inhibitor of metal-containing enzymes) has no inhibitory effect on the respiration. Barron (1951) considered that *p*-chloromercuribenzoate is a much more specific inhibitor for —SH containing enzymes than iodoacetate. It is quite evident from Fig. 4 that *p*-chloromercuribenzoate at M/70,000 exerted a marked effect on the oxygen uptake of the aerial mycelium. Final proof of the presence of —SH containing enzymes is obtained if the inhibition by known —SH inhibitors is reversed by certain —SH containing compounds; of these, glutathione is the most effective (Barron, 1951). All attempts to annul the inhibitions produced by *p*-chloromercuribenzoate by means of glutathione were unsuccessful.

Accumulation of keto bodies in the presence of pure oxygen in respiration experiments

Throughout the work with a pure oxygen gas-phase in Warburg experiments, it was consistently observed that keto bodies accumulated in the presence of oxygen. This was demonstrated by the use of the 2, 4-dinitrophenlyhydrazine test described by Jowett & Quastel (1937) and the ammonia nitroprusside test described by James & Norval (1938). The latter test is claimed to be more specific, especially for pyruvic acid (James & James, 1940), than the 2, 4-dinitrophenlyhydrazine reaction. Dickens (1946*a*) suggested that in the case of brain metabolism oxygen may poison the pyruvate oxidase system by attacking the essential —SH groups of the enzyme. In view of the accumulation of keto bodies (possibly pyruvic acid) during the respiration of the aerial mycelium of *Micromonospora vulgaris* in pure oxygen at 1 atm. pressure, it may well be that oxygen is here too causing a similar inactivation. It should also be mentioned that a similar accumulation of keto bodies was observed in the presence of the highly specific —SH inhibitor *p*-chloromercuribenzoate at M/70,000 and M/175,000 concentration.

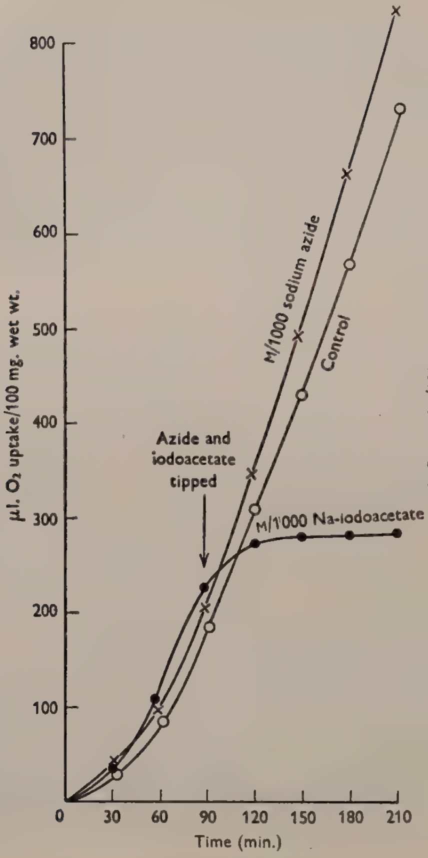

Fig. 3

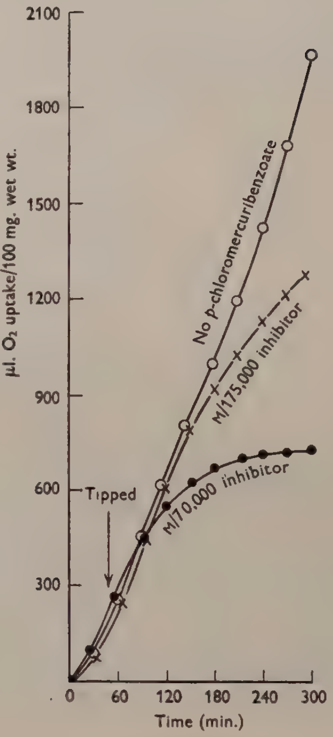

Fig. 4

Fig. 3. Effect of sodium azide and sodium iodoacetate on the respiration of the aerial mycelium of *M. vulgaris*. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with M/75 phosphate buffer (pH 7·0). Shaken for 5 min. in buffer on Microid shaker, subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg vessels. Further additions 0·5 ml. phosphate buffer + 1·5 ml. CPS + 0·5 ml. distilled water or 0·5 ml. 0·005 M-sodium azide or 0·005 M-sodium iodoacetate in side arm; side arm contents tipped after 90 min. Gas phase: air.

Fig. 4. Effect of *p*-chloromercuribenzoate on the oxygen uptake of *M. vulgaris* (aerial mycelium) at 60°. *M. vulgaris* (strain H); growth from liquid CPS medium after 2 days at 60° washed once with M/75 phosphate buffer (pH 7·0); shaken for 5 min. in buffer on Microid shaker and subsequently filtered through sterile glass wool. 75 mg. (wet weight) of surface growth remaining on glass wool weighed into Warburg flasks. Further additions 0·5 ml. phosphate buffer + 1·5 ml. CPS and various volumes of M/7000-*p*-chloromercuribenzoate in side arm to give final concentrations of M/70,000 or M/175,000. Distilled water added to give final volume of 2·5 ml.

DISCUSSION

Very little work seems to have been done on the production and metabolism of bottom and surface growths of aerobic micro-organisms in pure oxygen at one atmosphere pressure. Darby & Goddard (1950) found that in pure oxygen there was no difference in the rate of respiration of *Myrothecium verrucaria* in Warburg vessels as compared with that in air. As they grew their organism on a rotary shaker their material would have produced very little (if any) aerial mycelium. In the present work also, the growth and respiration of vegetative bottom growth was not adversely affected by the presence of pure oxygen (see Fig. 1; and growth experiments). On the other hand, the actual production of aerial mycelium was markedly suppressed by oxygen while the oxygen uptake of aerial mycelium was inhibited by the presence of pure oxygen at 1 atm. pressure. This gives rise to the question: does the physiology of the aerial mycelium vary from that of the vegetative growth? That the aerial growth of *Micromonospora vulgaris* is likely to be physiologically different from the vegetative growth is supported by the differences in morphology and the presence of lipid staining material in the former, which is absent from the latter (Erikson, 1952).

As regards the mechanism of inhibition, in many ways it parallels that described by Dickens (1946*a*) for the variations of brain tissue metabolism brought about by high oxygen pressures. Thus, inhibition increases with time and is irreversible. On the other hand, protection by divalent metals against inhibition as demonstrated by Dickens could not be shown to take place with *Micromonospora vulgaris*. It is possible, however, that the protective action of these metals might have been masked in the presence of the complex substrate mixture (CPS medium) which it was found necessary to use in Warburg experiments. The evidence obtained also suggests that the inhibition caused by pure oxygen may be connected with inactivation of thiol-containing enzymes. Thus, the oxygen uptake of the aerial mycelium is very sensitive to the highly specific —SH inhibitor *p*-chloromercuribenzoate and accumulation of keto bodies takes place in the presence of this inhibitor or of oxygen. Unfortunately, it has not been found possible to annul inhibition by *p*-chloromercuribenzoate by means of —SH compounds (Barron, 1951). Here again it is possible that the reaction of *p*-chloromercuribenzoate with —SH groups in enzymes functioning at 60° is somewhat different from its reaction with —SH enzymes functioning at more normal temperatures.

I am very grateful to Mrs D. Oxford for her advice and suggestions throughout this work and for supplying the material for the Warburg experiments. My thanks are due, also, to Miss Irene Taylor and Miss M. Michael for technical assistance, and to Dr G. K. Fraser for his interest throughout the work.

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Factors affecting Production of Cellulose at the Air/ Liquid Interface of a Culture of *Acetobacter xylinum*

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SUMMARY: The cells in a developing culture of *Acetobacter xylinum* in a static liquid glucose medium were swept to the surface by the flotation of a submerged cellulose net. The net was probably pulled to the surface by adsorbed carbon dioxide given off by the metabolizing cells. The relatively high oxygen pressure at the aerated surface of a static liquid culture mediated the rapid production of cellulose in the surface layer. *A. xylinum* produced less cellulose in swirled than in static nutrient medium. Cellulose in the swirled medium occurred in the form of remarkable bodies, spherical, jointed or stellate in form, up to 10 mm. in size. The swirling favoured the overgrowth of the wild type organism by mutants which were specifically deficient in the ability to form cellulose. On the basis of the type of colonial growth and of the pellicle characteristics, two groups of *A. xylinum* mutants with an extreme deficiency in cellulose-forming ability and one with an intermediate degree of deficiency were differentiated.

An extracellular framework consisting of cellulose characterizes the growth habitat of *Acetobacter xylinum* (Franz & Schiebold, 1943; Aschner & Hestrin, 1946; Frey-Wyssling & Muhlethaler, 1946). The understanding of the morphogenesis of this remarkable structure is relevant to efficient technical use of *A. xylinum* as an oxidizing organism and as a cellulose source (Evans & Hibbert, 1947) and to the use of the organism as a model system in which physical and chemical aspects of cell-wall production can be examined outside the cell (Hestrin, Aschner & Mager, 1947; Muhlethaler, 1948, 1949; Hestrin, 1953; Hestrin & Schramm, 1954). In the present communication the mechanics of the flotation and thickening of a xylinum pellicle are considered and the effects of agitation on the expression of the cellulose-synthesizing activity and its heritable transmission are described.

METHODS

Observations on growth of the pellicle under conditions of static culture were conducted in glass cylinders. Agitation of cultures was effected by swirling them in conical Erlenmeyer flasks with the aid of a Ross-Kershaw shaking apparatus maintained in a thermostat. The medium was inoculated with one-twentieth of its volume of the liquid phase of a starter culture. Loose cotton-wool stoppers which did not unduly obstruct oxygen supply were employed. The temperature in all experiments was 30°.

The liquid medium was of the following composition (%, w/v): glucose 2·0, Bactopeptone (Difco) 0·5; yeast extract (Difco) 0·5; initial pH value 6. A solid medium contained also 2·5 % of agar.

To determine the total fresh weight of a pellicle, it was lifted from the surface of the culture, hung freely on a glass peg for 15 min. to drain away the excess water, and weighed. The pellicle was then gently stirred with a large volume of distilled water for 90 min. to remove soluble material, and again weighed after drying at 80° over P_2O_5 *in vacuo*. Cellulose was determined by the method previously described (Schramm & Hestrin, 1954).

RESULTS

The flotation of the pellicle

An inoculum of *Acetobacter xylinum* added to liquid medium formed a diaphanous submerged net (Muhlethaler, 1948; Kaushal, Walker & Drummond, 1951). The culture fluid on the 1st day after inoculation, and before the formation of a visible surface pellicle, was somewhat turbid. Under static conditions in a shallow-layer liquid culture, a visible pellicle was present on the surface of the liquid after 2 days. The medium had cleared at this stage, the diaphanous net having risen to the surface with most of the bacteria enmeshed in it. The clearing of the medium at the same time as the appearance of the surface film suggested that a special mechanism had swept the cells into the surface layer. It was shown that the xylinum cells when acting on glucose mediate a slow evolution of carbon dioxide. A washed suspension of the cells shaken under oxygen at 30° in 4% glucose solution in 0·02 M-citrate (pH 6·4) formed carbon dioxide at a sustained rate of approximately 0·44 μmole/mg. cells (dry weight)/hr. as determined manometrically by Warburg's indirect method. Any gas evolved by a growing culture in an undisturbed liquid nutrient medium would accumulate on the surfaces of the cellulose fibrils; sometimes macroscopic bubbles of gas were detected in the submerged mesh. The gas probably caused the flotation of the net, and the net presumably pulled the bacteria to the surface.

The thickening of the pellicle

Synthesis of cellulose from glucose by a suspension of non-proliferating bacteria is a function of oxygen supply (Hestrin *et al.* 1947). The rate of cellulose production was negligible under nitrogen, about one-half optimal under air and approached closely the optimum rate under 100% oxygen in a reciprocally shaken suspension of cells in glucose solution. A relatively rapid increase of cellulose might thus be expected to take place as a result of the concentration of cells of a growing culture in the aerated surface zone. In a growing culture, moreover, the oxygen supply in the surface layer would mediate increase of the mass of the cells and of cellulose-forming enzyme and thus further augment cellulose production. Within certain limits, moreover, the system would be a self-accelerating one, since the increase of the cellulose content of the incipient surface film would serve to facilitate the retention of the cells in the surface.

The yields of cellulose found after 2- and 5-day periods of growth under air in static culture are illustrated by the data shown in Table 1. The yields of cellulose from the static cultures demonstrate the time lag preceding the colonization of the liquid surface and the dominant role of the area of the surface exposed to the gas phase in conditions of static culture. In agitated cultures, on the other hand, the total volume of the medium was the factor which determined the amount of cellulose obtained.

The observations just summarized confirm the view that the localization of cellulose production at the surface of the static liquid culture was determined by the oxygen pressure. It need not be assumed that mechanical and electrical vector fields in the surface played a causal role in the polymerization, although these might play a part in the secondary extracellular aggregation and crystallization of the cellulose molecules (cf. Franz & Schiebold, 1943; Aschner & Hestrin, 1946; Frey-Wyssling & Muhlethaler, 1946; Muhlethaler, 1948, 1949; Kaushal *et al.* 1951; Ranby, 1952*a*, *b*).

Table 1. *Influence of growth time, volume of medium and agitation on the production of cellulose by* A. xylinum *growing on liquid glucose medium*

The glucose medium was inoculated with 1/150th of its volume of fluid culture and distributed into conical flasks (50 ml.). Technique of cultivation and analytical procedure were as described in the section 'Methods'.

Period of incubation (days)	Volume of medium (ml.)	Conditions of growth	Total wt. of pellicle (mg.)		Yield of cellulose (mg.) (b)	Non-cellulose dry weight of pellicle (mg.) ($c=a-b$)	Non-cellulose: cellulose ratio (c/b)
			Wet	Dry (a)			
2	30	Static	419	—	2·10	—	—
	15	Static	432	5	1·78	3·2	1·8
	30	Swirled	—	—	0·84	—	—
	15	Swirled	—	—	0·45	—	—
5	30	Static	2680	34	13·2	20·8	1·6
	15	Static	2673	32	11·7	20·3	1·7
	30	Swirled	—	—	4·80	—	—
	15	Swirled	—	—	2·60	—	—

Cellulose production being thus a function of the oxygen pressure, it was expected that the increase of the mass of cellulose in a pellicle would occur principally in the upper part of it. The underpart of the pellicle would be pushed by this process progressively downwards into zones of decreasing oxygen pressure and lesser activity. This was experimentally verified as follows. Particles of cork were added to a xylinum culture on the same day as the inoculum. The particles floated on the surface and were incorporated within the forming pellicle. When the pellicle later increased in thickness, the cork particles remained fixed within the relatively transparent underpart of the pellicle, thus showing unambiguously that the accretion of cellulose had occurred principally in the upper part of the pellicle.

Whereas the upper part of the mature pellicle was generally opaque and quite firm, the under part was relatively transparent and soft, and frequently supported a fragile transparent sac which hung loosely from the undersurface. Kaushal *et al.* (1951) suggested that the relative transparency and lack of rigidity of the underpart of a pellicle might be indicative of a greater proportion of short and imperfectly oriented nascent chains of cellulose. However, when the cells of an opaque and firm pellicle are dissolved by storage in dilute sodium hydroxide solution, the recovered cellulose gel is highly transparent and soft. Yet the appearance of this purified product under the electron microscope, its chemical properties, X-ray diffraction diagram and infra-red absorption spectrum, are all those of a high-polymer crystalline cellulose (for example, see Ranby, 1952*a*; Forziati & Rowen, 1951).

The underpart of the pellicle has been shown above to be the oldest part. Thus the presence in this part of a higher proportion of short cellulose chains or of less well oriented chains appears to be rather unlikely, and some other explanation for the special properties displayed by this part may be desirable. It should not be overlooked that while the cellulosic extracellular gel component of the pellicle contributes the bulk of the gel volume, the dry weight of the pellicle is largely due to the bacteria in it (Table 1). It is therefore apparent that physical properties of a pellicle such as its opacity, rigidity, tensile strength and specific gravity could be functions of the condition and relative proportion of bacteria of the pellicle, and need not depend only on the nature, concentration and disposition of the cellulose chains in the extracellular gel fraction.

Influence of agitation on the production of cellulose

Since the rapid production of cellulose on the surface of a static liquid culture was determined by the relatively high oxygen pressure at the surface, it was anticipated that cellulose production in an agitated aerated liquid would take place throughout the volume of the medium. In fact, when the *Acetobacter xylinum* was transferred from static liquid culture to a swirled medium, it grew and synthesized cellulose. However, the pattern of the growth of cells and of the production of cellulose in the swirled medium showed important departures from the pattern in static liquid cultures. (*a*) The cultures in the swirled medium formed much less cellulose (Table 1). (*b*) The cellulose in the swirled cultures occurred in the form of remarkable macroscopic bodies (Pl. 1, fig. 1) in size up to *c.* 10 mm. diam. and spherical, jointed or stellate in form, resembling in appearance but exceeding in size the bodies of cellulose which are also formed by freeze-dried *A. xylinum* when it is swirled in buffered glucose solution. (*c*) Both at first transfer when the growth was allowed to continue for more than three days, and in swirled subcultures, the cells growing in swirled medium gradually lost ability to form cellulose and assumed a diffuse growth habit. To minimize the possibility that the late diffuse growth had been caused by contamination, the stock was purified by repeated plating on glucose agar. Four single-colony isolates from 4-day agar cultures all showed normal pellicle-forming ability in static liquid medium. In swirled cultures they showed the same behaviour as the original stock. The clones were transferred in swirled medium serially at intervals of 72 hr. With increase in the number of transfers, the growth in every case became more diffuse and there was a gradual diminution and finally disappearance of cellulose production (Pl. 1, fig. 2). The same observation was also made when the experiment was repeated with a strain of *A. xylinum* which had been placed at our disposal by Prof. M. Aschner.

Isolation and classification of mutants deficient in cellulose-forming ability

In a static liquid medium a floating pellicle insures an abundant oxygen supply to the cells. In a swirled liquid, on the other hand, the cells need not be floated by the pellicle in order to obtain oxygen and actively proliferating forms which were deficient in cellulose-forming ability might thus gain ascendancy. The view that the decline of cellulose production in swirl-culture is associated with overgrowth of the culture by cellulose deficient mutants was supported as follows.

A swirl-culture of fourth transfer, derived from a single-colony isolate of the *Acetobacter xylinum* stock, was plated on agar, and the properties of the isolated clones studied. On the basis of the types of colonies grown on agar (Pl. 2, fig. 3) and of the properties of the pellicles formed by the cultures on static liquid glucose medium (Table 2), the cultures were classified as wild-type or mutant. The mutant cultures were subclassified into three groups, two of them (celluloseless I and II) with extreme or absolute deficiency in cellulose-forming ability, and one with an intermediate degree of deficiency. All these mutants displayed characteristic Acetobacter properties, including growth in the form of rods of varying length, capacity to catalyse restricted oxidations with formation of acetic acid from ethanol and of acids from glucose, and formation of a zoogleal film on liquid medium. Since the zoogleal films which are formed by the 'celluloseless' mutants on the surface of liquid nutrient medium are fragile and dissolved by hot 4% sodium hydroxide, the cementing substance of these films is probably a substance other than cellulose.

Table 2. *Characteristics of surface pellicle of wild-type and cellulose-deficient mutants of* A. xylinum

Cultures were grown in static liquid glucose medium in test tubes and characterized on the 7th day.

	Properties of pellicle		
Organism	Thickness (mm.)	Coherence in 4% NaOH	Coherence when agitated gently
Wild-type	5	Unchanged	Unchanged
Intermediate	1*	Unchanged	Unchanged
Celluloseless I	1	Dispersed	Flakes
Celluloseless II	1	Dispersed	Dispersed

* Frayed pellicle with opaque patches.

The good growth rates of the mutants and their oxidative activities support the view that the states of deficiency of the mutants represent a specific lesion in the apparatus of cellulose synthesis rather than a generalized loss of metabolic vigour. The degree of the cellulose deficiency of the cultures was stably maintained in static liquid medium through ten serial transfers. When the wild-type was grown in mixed culture with any one of the mutants on a static liquid medium from a mixed inoculum containing a large preponderance of the mutant (0·05 ml. wild-type culture + 2·0 ml. mutant culture as mixed inoculum for 10 ml. medium), the wild-type rapidly gained the ascendancy. After a few transfers in static growth conditions, the mixed culture displayed the phenotypic characteristics of the wild-type, namely a cellulosic surface pellicle with clear underlying fluid. Therefore, the observed absence of a cellulose pellicle in the pure cultures of the mutants can be interpreted as affording evidence that genetic reversion to the wild-type is relatively rare. The observations are in agreement with the conclusion that the decline of the ability of *Acetobacter xylinum* to produce cellulose can occur by way of mutation in one or more steps. In view of the potential usefulness of the xylinum mutants as biochemical tools, it will be of particular interest to identify the enzyme lesion in the mutants more definitely and to ascertain whether the xylinum mutant strains accumulate a cellulose precursor.

The authors are grateful to Prof. M. Aschner for kind advice. Part of the work describing the isolation of mutants was carried out by one of us (M.S.) in work leading to the degree of Ph.D.

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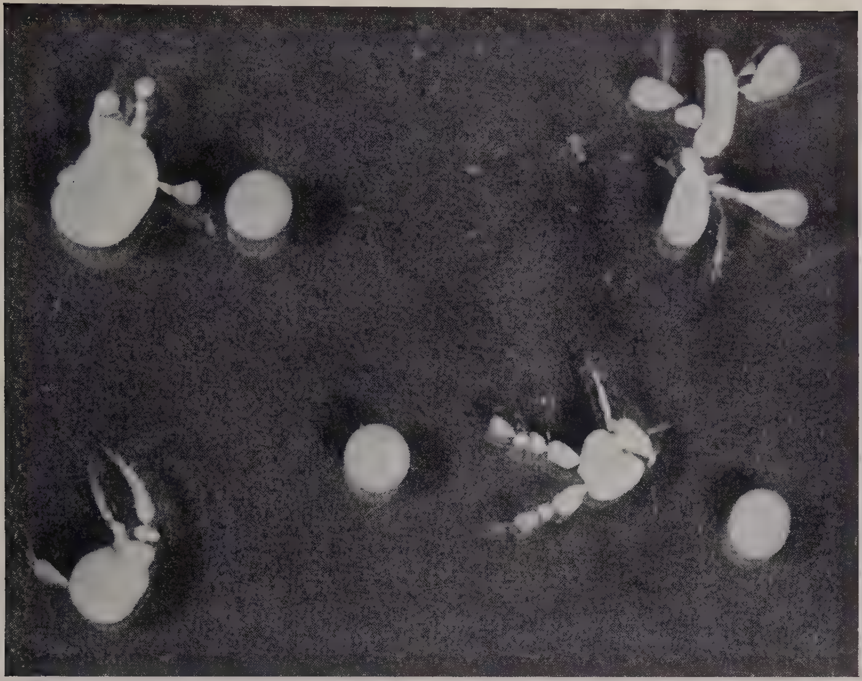


Fig. 1

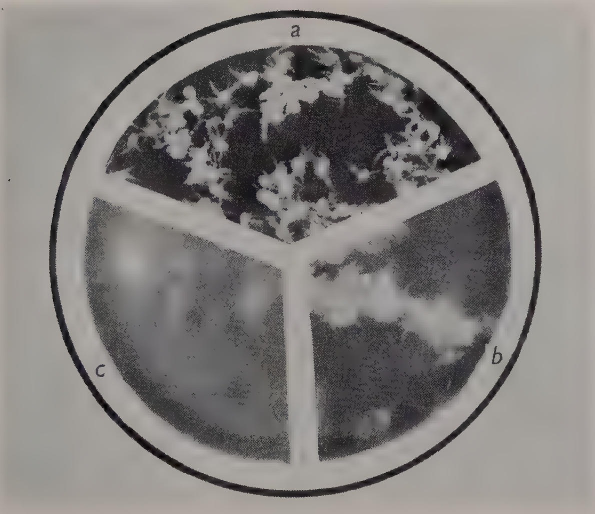
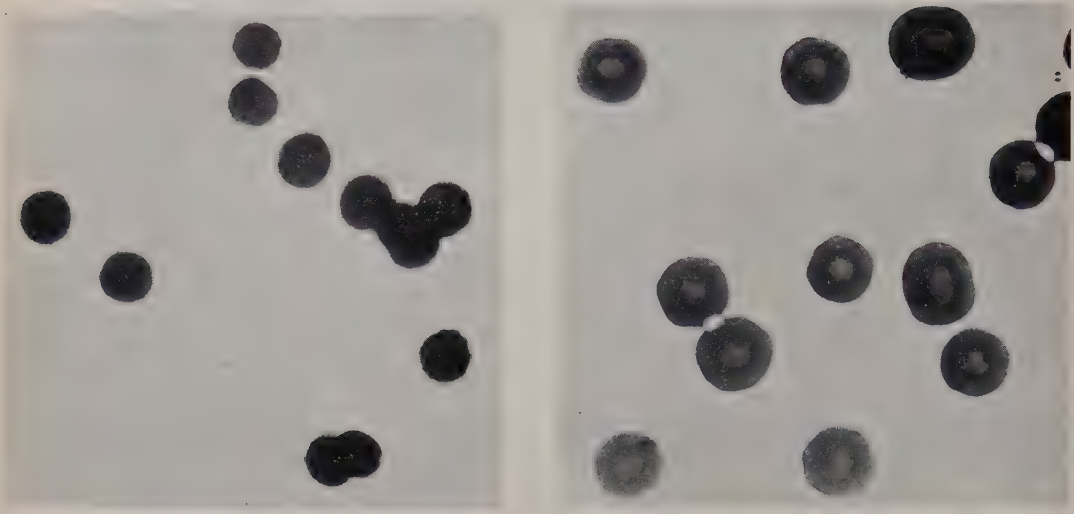


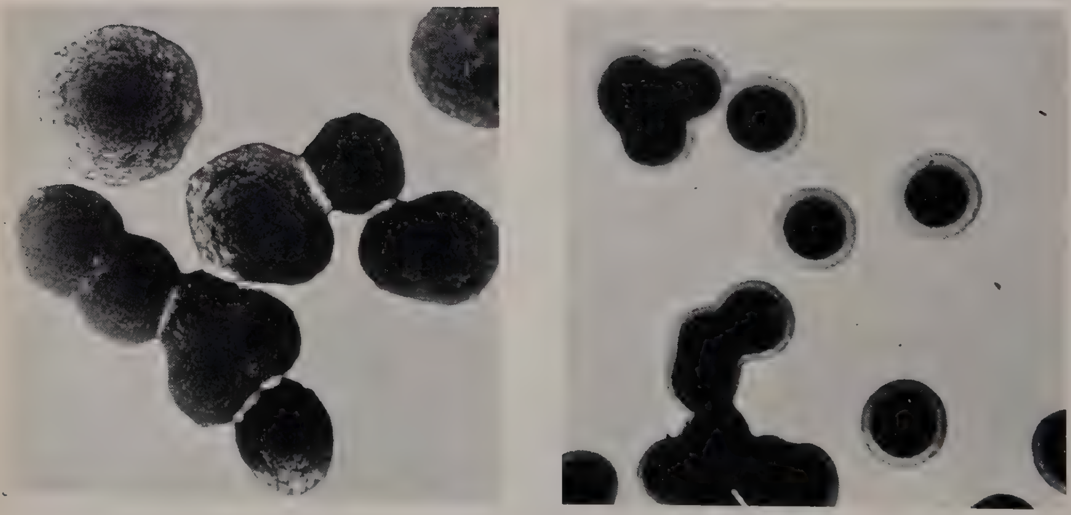
Fig. 2

M. SCHRAMM & S. HESTRIN—PRODUCTION OF CELLULOSE BY *A. XYLINUM*. PLATE 1



Quadrant 1

Quadrant 2



Quadrant 3

Quadrant 4

M. SCHRAMM & S. HESTRIN—PRODUCTION OF CELLULOSE BY *A. XYLINUM*. PLATE 2

EXPLANATION OF PLATES

Plate 1

Fig. 1. Spherical and jointed bodies formed by *Acetobacter xylinum* on transfer from static to swirled growth medium. ×4·5.

Fig. 2. Gross appearance of a swirl-culture of *A. xylinum*. Sectors *a*, *b* and *c*, are typical fields from photographs of a swirl-culture of the 1st, 2nd and 3rd transfer respectively. ×1.

Plate 2

Fig. 3. Colonies of *A. xylinum* wild-type and of mutants deficient in the ability to produce cellulose. The colonies on glucose agar were photographed, after 4 days of growth, as observed under the microscope by transmitted light. ×22. For characteristics of pellicles formed on liquid glucose medium see Table 2. Quadrant 1: colonies of wild-type; characteristically dwarf and heavily opaque. Quadrant 2: colonies of a mutant with intermediate deficiency in cellulose-forming ability; showing less opacity than the wild-type and a relatively bright central zone. Quadrant 3: colonies of the mutant designated celluloseless I; characteristically rough. Quadrant 4: colonies of mutant designated celluloseless II; with characteristic translucent outer rim.

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The Supposed Heat Stability of some Bacterial Enzymes: a Correction

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Oliver (1952) reported observations which he interpreted as indicating that decarboxylase systems of some lactic streptococci and some coliform organisms were relatively stable towards heat and chemical disinfectants. A recent check on the results has shown that the method used for estimating the carbon dioxide formed by a culture during a given period was faulty; the conclusions drawn as to the stability of the enzyme systems were therefore fallacious.

In the original experiments, cultures were grown in 100 ml. medium contained in a 150 ml. conical flask which was connected by a short length of 7 mm. diam. glass tubing to a second 150 ml. conical flask containing baryta. The apparatus, during incubation and during any heat treatment applied to the culture flask, was shaken only occasionally. In some experiments a vacuum was applied to the baryta flask in an effort to make sure that all the CO_2 formed in the culture was drawn into the baryta. In an examination of the method it became evident that under the conditions of Olivers's experiments carbon dioxide did not pass from the culture flask to the baryta flask as readily as had been assumed. A condition of supersaturation with respect to CO_2 often existed in the bacterial culture. Consequently the CO_2 formed by a living culture before the killing of the bacteria by heat or chemical disinfectants was only gradually evolved after the sterilizing procedure; hence the erroneous conclusion that the enzymes were heat stable.

The error of the method was made quite clear in a trial in which a solution of $NaHCO_3$ acidified with lactic acid to pH 3 was used in place of the bacterial culture in the apparatus. Under static conditions 25% of the theoretical amount of CO_2 passed over into the second flask in 24 hr. and was absorbed in the baryta; with constant gentle agitation 70% of the CO_2 was absorbed in 24 hr.; when a slow stream of CO_2-free air was bubbled through the solution, 100% of the CO_2 was absorbed in 1 hr.

Re-examination of the alleged stability of the decarboxylases using a technique in which the cultures were de-gassed by passage of CO_2-free air and the removed CO_2 absorbed in baryta in Pettenkofer tubes showed that the decarboxylase system of the lactic streptococci did not survive death of the bacteria. The enzyme systems of some coliform organisms did, however, show some evidence of heat stability, although much less than that claimed by Oliver.

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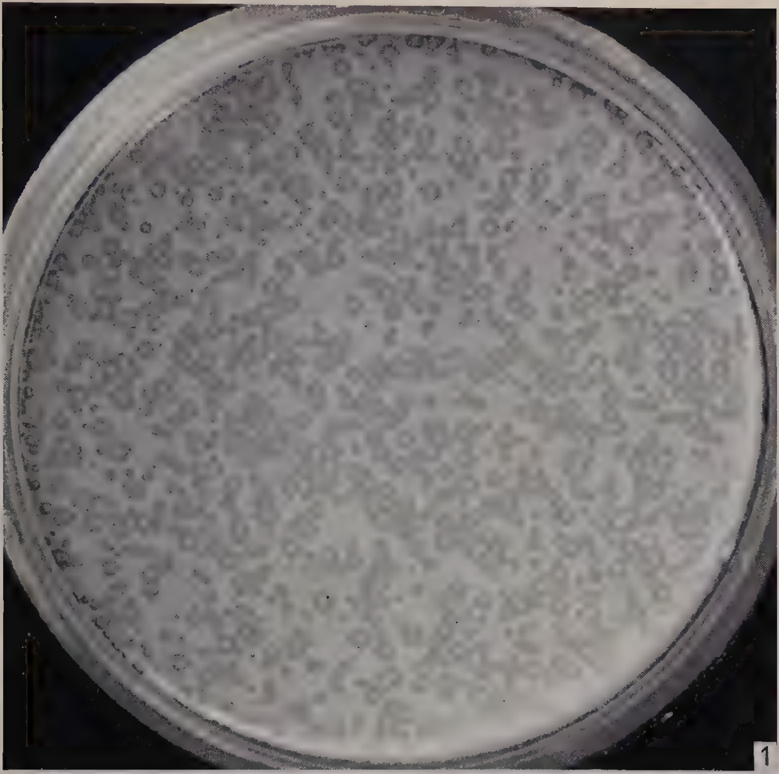

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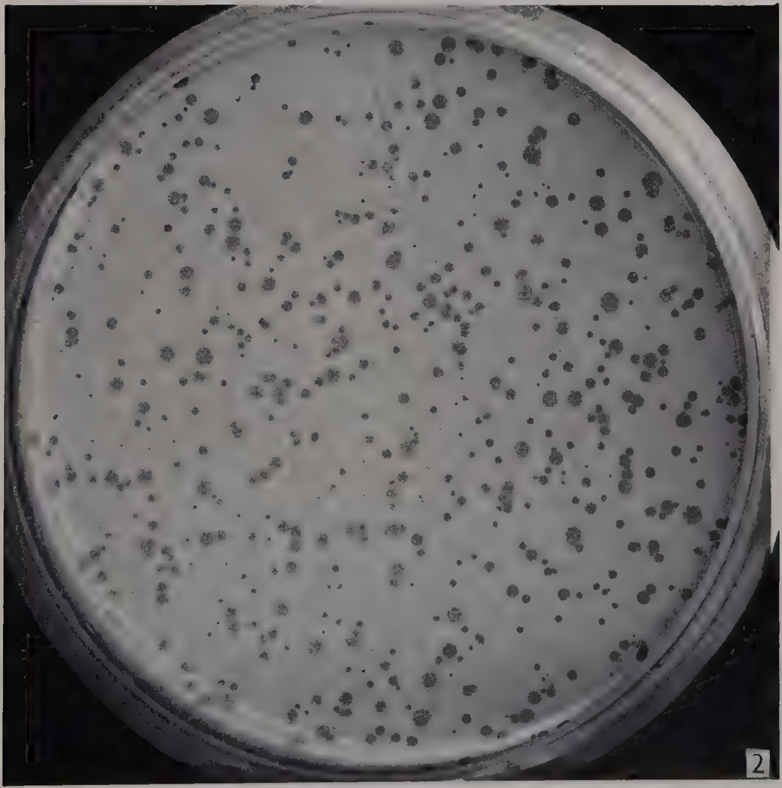

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Serological Subdivisions among the Lancefield Group H Streptococci

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SUMMARY: Seventeen members of Lancefield group H streptococci were separated into two 'precipitin types', by a series of absorption precipitation experiments. Representative strains of *Streptococcus sanguis* types I and I/II were found to be serologically identical with the majority of the strains designated as members of group H types 2 and 1 respectively. *S. sanguis* type II was considered not to possess the group H group-specific substance.

The Lancefield group H streptococci were first identified by Hare (1935) who isolated them from apparently normal human throats. Two of the strains isolated by Hare were further investigated by Sherman (1937) and other members of this group were examined by Hehre & Neill (1946), Dodd (1949) and Porterfield (1950). In 1946, Loewe, Plummer, Niven & Sherman isolated a streptococcus, later called *Streptoccocus sanguis* (White, 1944), from the blood of 39 % of patients with subacute bacterial endocarditis whom they examined. The characteristics of forty-two of these strains were investigated by Washburn, White & Niven (1946), and by White & Niven (1946). Although these workers were unable to isolate any strains of *S. sanguis* from the throats of normal patients, both Dodd (1949) and Porterfield (1950) considered that some strains of *S. sanguis* were members of Lancefield group H. Dodd (1949) claimed that the results of absorption precipitation experiments indicated the presence of at least five antigenically different types within the members of group H, and included *S. sanguis* types I and II within this group. Porterfield (1950), while agreeing that *S. sanguis* type I was a member of group H, found that the third type, namely *S. sanguis* type I/II, also possessed the group H specific substance. He was, however, unable to demonstrate the group H antigen in *S. sanguis* type II. In view of the serological relationship of *S. sanguis* with Lancefield group H streptococci and the frequent isolation of the members of this group from the throats and mouths of normal patients (Farmer, 1953), it was considered of interest to investigate further the characteristics of the members of the group H streptococci. Accordingly, the cultural and biochemical characteristics of 24 strains were investigated, and seventeen strains were examined serologically by a series of absorption precipitation experiments.

EXPERIMENTAL

Source of organisms. Three of the strains examined came from the original collection of Prof. Hare, and were kindly provided by Dr Fry, together with 1 other strain; 2 other strains came from the blood of patients with subacute bacterial endocarditis and 1 from the mouth of one of these patients; single representatives of *Streptococcus sanguis* types I, II and I/II came from the

National Collection of Type Cultures (NCTC), where they were numbered 7863, 7864 and 7865 respectively, and the remaining strains were isolated in Cambridge from the saliva or gingival sulci of apparently healthy patients.

Antigen extracts. The acid and formamide extracts of all strains were prepared from the growth in 50 ml. Hartley digest broth containing 1 % glucose, using the methods suggested by Lancefield (1933) and Fuller (1938). Extracts were tested for ring-precipitate formation, in specially prepared tapering tubes modified from Lancefield (1938), using two group H grouping sera, one prepared by Dr Fry with the strain Blackburn and labelled H_4, and the other prepared in the Burroughs Wellcome Research Laboratories and labelled no. 6244. Only those extracts which formed good precipitates at room temperature in about 10 min. were regarded as being positive.

Sera. The sera used in the absorption precipitation experiments were prepared in rabbits. Immunization was by means of formalin-killed suspensions (Lancefield, 1933) with four or six series of four injections of 1 ml. each, or live suspensions starting with small doses of 0·1 ml. and doubling the dose on alternate days up to 1·6 ml. on the 9th day. Suspensions of organisms were made to have the opacity of an 18 hr. glucose broth culture. Rabbits injected with killed suspensions were bled on the 5th day after the last injection, while those injected with live organisms were bled on the 7th day after the last injection. The sera which formed satisfactory precipitates when tested with the extracts of the homologous strain were kept and stored at −20° without preservative.

The method used for the absorption of the sera was modified from that of Lancefield (1933). The sera were mixed with an equal volume of packed heat-killed cells and incubated in a water-bath at 37° for three-quarters of an hour. Those sera which formed precipitates after absorption when tested against any of the extracts used in the experiment were re-absorbed and re-tested. All absorptions were controlled by parallel absorptions using the cells of a heterologous Lancefield group G strain. The cultural and biochemical characteristics were investigated by the methods reported elsewhere (Farmer, 1951).

RESULTS

Serological

Extracts of the 17 strains formed strong precipitates when tested with the 2 group H grouping sera. Sera prepared in rabbits with 6 of these strains showed no cross-reactions with extracts of members of Lancefield groups A to K and L to O, but showed strong reactions with those of known members of group H.

Preliminary absorption precipitation experiments with some of the sera indicated that not all the strains considered were serologically identical. To investigate these results the sera prepared from 6 group H strains and the 2 group H grouping sera were absorbed with most of the available group H strains and the resulting absorbed sera were then tested against the acid extracts of the 17 strains. It was found that the sera prepared with the strains labelled 187 and Challis and the Blackburn antiserum (H_4) were completely absorbed by 7 strains, namely 187, Challis, Blackburn, Hatchman, 14, Morrison and Channon (Table 1). These strains, together with 5 others whose extracts reacted in a similar way, were classified as group H type 1. After absorption with the strains B and 44 these sera still formed good precipitates with the extracts of the above 12 strains and accordingly the strains B and 44, together with three others, were classified as group H type 2.

Table 1. *A summary of the results of absorption precipitation experiments using sera prepared with the group H streptococci labelled Blackburn, Challis and* 187.

	Sera prepared with group H streptococci Blackburn, Challis and 187		
Acid extracts of streptococci	Unabsorbed	Absorbed by group H type 1 strains (7 strains)	Absorbed by group H type 2 strains (2 strains)
Group H type 1 (12 strains)	+++	–	++
Group H type 2 (5 strains)	+++	–	–

Table 2. *A summary of the results of absorption precipitation experiments using serum prepared with the group H strain labelled Hatchman*

	Serum prepared with strain Hatchman			
Acid extracts	Unabsorbed	Absorbed with group H type 1 strains (excluding Blackburn, Challis and Channon)	Absorbed with strains Blackburn, Challis and Channon	Absorbed with Group H, type 2 strains B & 44
Group H, type 1 (excluding strains Blackburn, Challis and Channon)	+++	–	+++	+++
Blackburn, Challis and Channon	+++	–	–	–
Group H type 2	+++	–	–	–

Not all the sera prepared with the type 1 strains produced the same results as each other. That prepared with the strain Hatchman was completely absorbed by 4 type 1 strains, but was incompletely absorbed by the strains Blackburn, Challis and Channon (see Table 2). The acid extracts of these 3 strains differed in their reactions from those of the other type 1 strains in that they failed to form precipitates with this serum after it had been absorbed by the type 2 strains, B and 44. The serum prepared with strain 14 produced similar results to those recorded in Table 2, but in this case the extracts of strains Blackburn, Challis and Channon also formed strong reactions after the serum had been absorbed with the type 2 strains B and 44.

The sera prepared with two of the strains classified as type 2, namely B and 44, were each completely absorbed by the cells of these 2 strains and also by the cells of the 3 others previously classified as type 2 (see Table 3). These 2 sera did not, however, show identical reactions when absorbed with the type 1 cells. Anti-B serum was incompletely absorbed by all the type 1 cells while the anti-44 serum, which did not form very strong reactions even when unabsorbed, was apparently completely absorbed by 4 of the 7 type 1 strains tested, namely Blackburn, Challis, Channon and Morrison. The Burroughs Wellcome serum, prepared with a strain labelled CN 880, was completely absorbed by the cells of all the group H strains classified into both types 1 and 2.

Table 3. *A summary of the results of absorption precipitation experiments using the serum prepared with group H type 2 streptococci labelled B.*

Acid extracts	Serum prepared with strain B Unabsorbed	Absorbed with group H type 1 strains (7 strains)	Absorbed with group H type 2 strains (5 strains)
Group H type 1 (12 strains)	+++	−	−
Group H type 2 B and 44	+++	++	−
89, 54 and K	++	+	−

The separation of strains into types 1 and 2 was not so clearly defined when formamide extracts of cells were used. The formamide extracts of type 1 cells failed to react with the homologous sera after absorption with the type 2 cells, but those of the members of type 2 still reacted with the absorbed homologous sera in the same way as their acid extracts. In all cases lowering the temperature of formamide extraction to 134° or 100° failed to affect the results, but generally reduced the quality of the precipitates formed with the unabsorbed and the absorbed sera.

Incubation of the acid extracts of 4 members of type 1 and 2 of type 2 with 2 %, 4 % or 8 % (v/v) trypsin (Allen & Hanburys) over a period of 24 hr. (method of Lancefield, 1934), failed to alter the reaction with any of the absorbed sera.

Relationship of Streptococcus sanguis *to Lancefield group H Streptococci*

When acid extracts of the available strains of the 3 types of *Streptococcus sanguis* were tested against the group H antisera, *S. sanguis* type I and type I/II both formed strong precipitates (Table 4). *S. sanguis* type II formed moderately strong precipitates with most of these sera but, as found by Porterfield (1950), it did not react with the Burroughs Wellcome serum or with that prepared with the strain Challis. With the previously described absorbed group H sera (Tables 1, 2 & 3), the extract of *S. sanguis* type I showed the same reactions as the group H type 2 strains, while *S. sanguis* type I/II reacted in the same way as the majority of the group H type 1 strains. All the sera prepared with the group H type 1 strains were completely absorbed by the cells of *S. sanguis* type I/II, and those prepared with group H type 2 strains were completely absorbed by the cells of *S. sanguis* type I. *S. sanguis* type II did not remove any precipitating antibodies from these sera except those for its own antigens.

Table 4. *The precipitates formed when the acid extracts of the three types of* Streptococcus sanguis *were tested against sera prepared with members of Lancefield group H streptococci*

	Serum prepared with the following strains							
Acid extracts	Burroughs Wellcome group H	Challis	Blackburn	Hatchman	187	14	B	44
S. sanguis type I	++	+++	+++	+++	+++	+++	+++	+++
S. sanguis type II	–	–	++	++	+++	+++	++	++
S. sanguis type I/II	++	+++	+++	+++	+++	+++	+++	+++

Sera were prepared with the three types of *Streptococcus sanguis* and the results of the absorption precipitation experiments of Washburn, White & Niven (1946) were confirmed. Reciprocal absorption precipitation experiments were then carried out with the group H strains. The results (Table 5) confirmed the identity of *S. sanguis* type I with the members of group H type 2 and of *S. sanguis* type I/II with the majority of the members of group H type 1. The *S. sanguis* type II antiserum showed a poor reaction with its own extract after absorption with group H type 1 cells. The extracts of *S. sanguis* types II and I/II and the group H type 1 strains produced good reactions with this serum after absorption with the group H type 2 cells.

Cultural and biochemical tests

The results of cultural and biochemical tests on the 24 group H streptococci (Farmer, 1953) were very similar to those reported by Porterfield (1950). Owing to the disproportionate numbers of strains in the two types, it was considered of little value to record the characteristics of each type separately, but no one characteristic, nor combination of characteristics, could be found to be related to the serological subdivisions.

DISCUSSION

From the results of the absorption precipitation experiments with 17 strains of Lancefield group H streptococci, it was apparent that this group of organisms was not serologically homogeneous. Many of the strains examined, particularly those isolated from mouths, formed unstable suspensions, and it was not possible to test the results by absorption agglutination experiments. It seemed probable, however, that the serological differences between the strains

Table 5. *The precipitates formed when the acid extracts of streptococci of group H types* 1 *and* 2 *were tested against the sera prepared with* Streptococcus sanguis *types I, II and I/II absorbed by the cells of the designated types. The precipitates formed with the extracts of the three types of* S. sanguis *are also recorded*

	Sera prepared with								
	S. sanguis type I			*S. sanguis* type II			*S. sanguis* type I/II		
		Absorbed with the cells of group H streptococci			Absorbed with the cells of group H streptococci			Absorbed with the cells of group H streptococci	
Acid extracts	Unabsorbed	Type 1	Type 2	Unabsorbed	Type 1	Type 2	Unabsorbed	Type 1	Type 2
Group H type 1 (12 strains)	+++	–	–	++	–	++	+++	–	++
Group H type 2 (5 strains)	+++	++	–	++	–	–	+++	–	–
S. sanguis type I	+++	++	–	++	–	–	+++	–	–
S. sanguis type II	++	–	–	++	+	++	++	–	–
S. sanguis type I/II	+++	–	–	++	–	++	+++	–	++

examined was due to the presence of different type antigens, and accordingly the strains were divided into two types.

Not all the strains classified into each type showed exactly similar results with each of the sera used. The three classical strains, Blackburn, Challis and Channon, did not seem to possess a type 1 antigen identical with that possessed by the other members classified within this type, but although they reacted in different ways from the other type 1 strains with some of the sera used, there did not seem to be sufficient evidence to classify them into a third type. Other similar differences in reactions were seen among the strains at present allocated to the two types; further investigations may therefore reveal further divisions among the streptococci sharing the group H antigen. The failure of trypsin to affect the extracts of selected members of each type suggests that the type antigens are probably not proteins.

The extracts of four other streptococci not included in these results, formed moderately good precipitates with most of the group H type 1 antisera, but, because they failed to react with either the type 2 antisera or the Burroughs Wellcome group H grouping serum, they were considered not to possess the group H antigen. It is of interest to note that they were able to absorb the serum prepared with *Streptococcus sanguis* type II completely and although reciprocal absorption precipitation experiments were not carried out, it is probable that they shared some of the antigens present in this strain of *S. sanguis*. The reciprocal absorption precipitation tests using sera prepared with the group H streptococci and the strains of *S. sanguis* confirmed the results of Porterfield (1950) that *S. sanguis* type I and I/II possessed the group H antigen. The results also showed that *S. sanguis* types I and I/II shared the type antigens of the majority of those strains allocated to group H types 2 and 1 respectively. The failure of *S. sanguis* type II to remove the precipitating antibodies from any of the absorbed group H sera, excepting those for its own extract, indicated that it probably did not possess the group H antigen.

It was found from the results of the investigation of the cultural and biochemical characteristics of 194 streptococci (Farmer, 1951, 1953), that the possession of no one physiological characteristic was a definite indication that the strain was a member of Lancefield group H, but that a high proportion of the members of this group possessed a combination of characteristics not usually found among the members of other Lancefield groups. This combination of characteristics was found by White & Niven (1946) to be possessed also by *Streptococcus sanguis* and included the ability to hydrolyse arginine, to grow on blood agar in the presence of 40 % (v/v), bile, to decolorize and grow in the presence of 0·1 % methylene blue in milk, to produce a detectable dextran from sucrose and to ferment inulin but not raffinose. There was no apparent relationship between the serological differences among the group H streptococci and their cultural and biochemical characteristics.

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Studies on the Metabolism of *Thiobacillus thiocyanoxidans*

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SUMMARY: The investigation of the metabolism of thiocyanate by pure cultures of *Thiobacillus thiocyanoxidans* was complicated by the fact that thiocyanate serves both as source of energy and as source of carbon and nitrogen. It was, therefore, difficult to separate oxidation from carbon dioxide fixation. The equation which best represented the resultant of these two processes was:

$$2KCNS + 5H_2O + 3O_2 = K_2SO_4 + (NH_4)_2SO_4 + CO_2 + (CH_2O).$$

Thiocyanate was first hydrolysed to cyanate and sulphide. Cyanate was further hydrolysed to carbon dioxide and ammonia and sulphide was oxidized to sulphate. The oxidation of sulphide approximated to the equation: $Na_2S + 2O_2 = Na_2SO_4$. Gas uptakes were lower than would be expected from this equation, although special precautions were taken to prevent loss of sulphide.

Thiosulphate was oxidized to sulphate according to the equation:

$$Na_2S_2O_3 + H_2O + 2O_2 = Na_2SO_4 + H_2SO_4.$$

Fixation of carbon dioxide after the oxidation of thiosulphate was shown, but the efficiency of energy utilization appeared lower than during the oxidation of thiocyanate.

Thioacetamide and thioacetic acid were oxidized to sulphate but this was believed to be preceded by a chemical breakdown to sulphide. Sulphite, metabisulphite, dithionite, dithionate, tetrathionate, trithionate, thiourea and cysteine were tested; no evidence was obtained of their utilization.

The presence of thiocyanate in gas works liquors led to early experiments on the biological oxidation of the compound. Sludges were activated by exposure to the compound until they were metabolizing 400 p.p.m. thiocyanate ion/24 hr. The products, ammonia and sulphate, appeared in amounts which suggested the equation: $NH_4CNS + 2H_2O + 2O_2 = (NH_4)_2SO_4 + CO_2$ for the reaction. A preliminary examination of the activated sludge showed the presence of many apparently autotrophic organisms, and it was suggested that the oxidation of thiocyanate supplied energy for autotrophic growth (Happold & Key, 1934). Further investigations of the bacterial flora were described by Happold & Key (1937). The autotroph was not isolated in pure culture until 1951 (Happold, Johnstone & Rogers, 1952); later it was re-isolated and its bacteriological characteristics described (Happold, Johnstone, Rogers & Youatt, 1954). Gleen (1949) studied thiocyanate metabolism by a soil-perfusion technique. No experimental details were published but he recorded that the same population could use thiosulphate and thiocyanate. He confirmed the production of ammonia and sulphate and suggested the preliminary hydrolysis of thiocyanate to cyanate and hydrosulphide. The aim of the present investigation was to examine further the metabolism of thiocyanate by pure cultures. Experiments with growing cultures were described by Happold *et al.* (1954); the present account is of experiments made with washed suspensions of organisms from pure cultures.

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METHODS

Growth and preparation of Thiobacillus thiocyanoxidans. The growth medium contained: Na_2HPO_4, 1·0 g.; KH_2PO_4, 0·6 g.; KCNS, 0·2 g.; $MgSO_4 7H_2O$, 0·02 g.; a trace of iron; distilled water to 1 l.; pH value between pH 7·0 and 7·2. Thirty to eighty litres were inoculated and incubated at 30° for 2–4 days. In a previous paper (Happold *et al.* 1954) the difficulty of estimating the degree of bacterial growth was described. This was due to the poor growth, the clumping of cells and the variable presence of sulphur particles. Generally, however, the cultures obtained after 2–4 days of incubation showed more than 90 % light transmission, measured with a Unicam D.G. Spectrophotometer in ½ in. tubes at 6500 A. The organisms were harvested in a heat-sterilized De Laval Centrifugal Separator, and after harvesting were washed and suspended in a M/15 Na–K phosphate buffer (pH 7). It should be noted that, although this phosphate concentration was recorded as inhibiting the growth of cultures (Happold *et. al.* 1954), phosphate concentrations between M/10 and M/100 showed no effect with washed cell suspensions. Some attempts were made to prepare crushed cells and cell extracts using the Hughes press (Hughes, 1951) or by shaking with glass beads.

Estimations. All colorimetric and turbidimetric measurements were made with a Unicam D.G. Spectrophotometer.

Potassium thiocyanate solutions were standardized against silver nitrate. Ammonia was estimated, after removal of cells, by the addition of Nessler reagent. Sulphate was estimated turbidimetrically after removal of cells, by the addition of solid barium chloride and comparison with freshly prepared standards. Colorimetric estimations of sulphide were made by Pomeroy's method (Pomeroy, 1936). This method did not appear to be entirely satisfactory and the results obtained were regarded as semi-quantitative. Sodium sulphide and sodium sulphite solutions were standardized by the addition of excess standard iodine and back titration with sodium thiosulphate. Sodium cyanate solutions were prepared by weight, and the ammonia production on hydrolysis was taken as an indication of the purity of the sample. Tetrathionate and trithionate were prepared by the method of Kurtenacker & Matejka (1930). Owing to the difficulty of preparing pure samples of tetrathionate, a second method was employed, namely the oxidation of thiosulphate by iodine.

Respiration experiments were made at 30° by the usual manometric techniques. Carbon dioxide production from cyanate was determined by allowing the reaction to occur in the absence of KOH and then freeing the dissolved carbon dioxide by tipping in hydrochloride acid at the end of the reaction. Carbon dioxide production from thiocyanate was determined by the difference in gas volume change in the presence and absence of KOH

(0·2 ml. 10 % KOH). Hydrochloric acid was tipped at the end of the reaction in flasks which contained no KOH.

Owing to the rapid loss of H_2S from neutral sulphide solutions the following technique was adopted for the addition of sulphide to flasks. The cell suspension was placed in one side arm of a two side arm flask and un-neutralized sodium sulphide was added to the other. The main compartment contained phosphate buffer and sufficient hydrochloric acid to neutralize the sulphide. No KOH was present. The sulphide solution was the last addition made to the flasks which were immediately attached to the manometers and transferred to the bath. The manometer taps were closed within a few seconds and the equilibrium time was kept as short as possible. It was necessary to record the initial reading before tipping in the sulphide as there was a release of H_2S on neutralization.

In all cases where a molar ratio of gas produced or absorbed to substrate metabolized was determined, the gas volume change with different amounts of substrate was measured. The results were plotted and the ratio, mole gas : mole substrate, was calculated from the slope of the best straight line. The ratios of the products of reaction were similarly determined.

Anaerobic experiments were made in Thunberg tubes. The introduction of KOH through the side arm before sampling was used in an attempt to decrease loss of H_2S to the air.

RESULTS

The metabolism of thiocyanate can be divided into several steps which were as far as possible studied separately. The experimental results are, therefore, divided according to the step with which they are concerned.

Hydrolysis of thiocyanate

This step can be represented as: $KCNS + H_2O = HCNO + KSH$, but a simple demonstration of this reaction was not achieved because any cyanate formed was further hydrolysed to carbon dioxide and ammonia. In anaerobic conditions approximately 50 % of the theoretical amount of sulphide was obtained experimentally; experimental difficulties, which included inhibition of the reaction by sulphide, loss of sulphide from solution and precipitation of metal sulphides on the cells, probably prevented the demonstration of higher yields by this method. In manometer flasks production of sulphide was observed under aerobic conditions when whole cells were exposed to 100 μg. cetyl trimethylammonium bromide per ml. or when cell extracts were used. Crushed cells did not show a complete loss of oxidizing ability, as the extracts did, but the rate of oxidation was much decreased.

The hydrolysis of cyanate

This step may be represented as: $NaCNO + 2H_2O = CO_2 + NH_3 + NaOH$. Ammonia and carbon dioxide were produced from cyanate. Cyanate in acid solutions decomposes to give ammonia and carbon dioxide so that the need to introduce acid to release dissolved carbon dioxide after bacterial hydrolysis was a disadvantage. However, production of ammonia and some production of carbon dioxide was demonstrated without the addition of acid. Table 1 shows the production of ammonia and carbon dioxide from different amounts of sodium cyanate.

Table 1. *The hydrolysis of cyanate*

Washed suspensions of *Thiobacillus thiocyanoxidans* grown on thiocyanate and suspended in M/15 K Na phosphate buffer (pH 7) were shaken in manometer flasks in air at 30°, with different amounts of sodium cyanate in the same buffer; final volume of liquid 3 ml. Carbon dioxide was determined as total gas volume released after acidification; ammonia was estimated as described in the text.

The molar ratio $NaCNO:CO_2:NH_3$ estimated graphically (see text); the ratio for this experiment was 1:1·08:1·1.

NaCNO added (μ mole)	Carbon dioxide produced (μ mole)	Ammonia produced (μ mole)
0	3·0	2·5
2	3·3	3·4
4	5·9	6·3
6	8·0	8·6
8	9·4	10·7
10	11·0	13·1

The oxidation of sulphide

The theoretical equation for the oxidation of sulphide was:

$$Na_2S + 2O_2 = Na_2SO_4.$$

The absorption of oxygen and the production of sulphate from oxidation of sulphide were determined by the methods described. Table 2 shows the result of one such experiment. The sulphide concentration could not be allowed to exceed M/600 Na_2S; above this concentration there was inhibition by sulphide, and sulphur rather than sulphate was found. Owing to this inhibitory effect of sulphide it was not possible to compare satisfactorily the rates of oxidation of sulphide and thiocyanate. However, over a short time interval with a low concentration of sulphide a rate was observed which was equal to the rate of oxidation of thiocyanate by the same suspension.

Table 2. *The oxidation of sulphide*

Washed suspensions of *T. thiocyanoxidans* grown on thiocyanate and suspended in M/15 K Na phosphate buffer (pH 7) were shaken in manometer flasks, in air, at 30°, with different amounts of sodium sulphide. Special precautions were taken to prevent loss of sulphide during equilibration and sulphate was determined at the end of the reaction (see text).

The molar ratio: $Na_2S:O_2:SO_4$ estimated graphically (see text) was 1:1·7:1·09.

Na_2S added (μ mole)	Oxygen absorbed (μ mole)	Sulphate produced (μ mole)
0·0	0	0
0·81	0·7	1·2
1·62	1·9	2·0
2·43	3·5	2·9
3·24	5·2	3·9
4·05	6·7	4·3

The production of ammonia and sulphate from thiocyanate

The equation which has been suggested for the oxidation of thiocyanate is:

$$2KCNS + 5H_2O + 3O_2 = K_2SO_4 + (NH_4)_2SO_4 + CO_2 + (CH_2O).$$

Ammonia was estimated in the flask contents when all gas uptake had ceased. Table 3 shows the ammonia yields from the metabolism of different amounts of thiocyanate. The yields were similar with or without KOH in the centre well. There was, therefore, no measurable assimilation of nitrogen even when carbon dioxide was also available. Thus, although the necessary conditions of growth were present, there could have been little growth in the short duration of the experiments.

Table 3. *The metabolism of thiocyanate*

Washed suspensions of *Thiobacillus thiocyanoxidans*, grown on thiocyanate and suspended in M/15 K Na phosphate buffer (pH 7) were shaken in air at 30°, with different amounts of KCNS. One series had 0·2 ml. KOH (10 %) in the centre well; to the other 0·2 ml. dil. HCl was added at the end of the reaction. Oxygen uptakes were determined in the first series and carbon dioxide production was found by difference between the two. Ammonia and sulphate were determined at the end of the reaction. The molar ratio

$$KCNS : O_2 : CO_2 : SO_4^= : NH_3$$

estimated graphically (see text) was 1:1·55:0·53:1·02:1·03.

KCNS added (μ mole)	O_2 absorbed (μ mole)	CO_2 produced (μ mole)	SO_4 produced (μ mole)	NH_3 produced (μ mole)
0	0·3	2·5	0	0
2	3·2	3·4	..	.
4	6·4	.	4·3	4·2
6	.	5·1	6·6	6·2
8	12·8	6·2	8·6	8·2
10	15·6	7·9	10·2	9·9

Sulphate was estimated in the flask contents at the end of oxygen uptake by suspensions which had been supplied with varying amounts of thiocyanate. The results of one such experiment are shown in Table 3.

Oxygen absorption for the oxidation of thiocyanate

During the oxidation of thiocyanate, carbon dioxide was produced by the initial hydrolysis of thiocyanate. It is possible that the organisms may be able to utilize some of this carbon dioxide before it leaves the cell and is absorbed by the KOH present in the centre well of the flasks. According to the equations which were previously suggested for the oxidation of thiocyanate (Happold & Key, 1934; Gleen, 1949) thiocyanate is oxidized with the uptake of 2 mole oxygen to one of thiocyanate. In Table 3 the ratio mole O_2 : mole KCNS was found to be 1·55 : 1. In other experiments the molar ratios were: 1·58 : 1, 1·62 : 1, 1·52 : 1, 1·62 : 1 and 1·45 : 1. These results are discussed later. The plotted curve for oxygen uptake during the oxidation of thiocyanate showed a short initial lag, presumably because the first step was a hydrolysis.

The production of carbon dioxide from thiocyanate

This measurement was necessarily made while oxidative reactions were taking place. From the autotrophic nature of the organism it was to be expected that some fixation of carbon dioxide would be found. The details of one experiment are given in Table 3. The ratios, mole O_2 : mole KCNS and mole CO_2 : mole KCNS in this experiment were 1·55 : 1 and 0·53 : 1 respectively. In two other experiments the molar ratios O_2 : KCNS : CO_2 were 1·62 : 1 : 0·65 and 1·45 : 1 : 0·58.

The oxidation of thiosulphate

The possibility that thiosulphate is an intermediate in the oxidation of thiocyanate is discussed later. The oxidation of thiosulphate may be represented: $Na_2S_2O_3 + H_2O + 2O_2 = Na_2SO_4 + H_2SO_4$. Table 4 shows the oxygen absorption and sulphate production from different amounts of thiosulphate. These were in close agreement with the theoretical equation. The oxidation of thiosulphate was linear from the time of addition and the rate was usually equal to or greater than the rate of oxidation of equimolar amounts of thiocyanate.

Table 4. *The oxidation of thiosulphate*

Washed suspensions of *Thiobacillus thiocyanoxidans* grown on thiocyanate and suspended in M/15 K Na phosphate buffer (pH 7) were shaken in air at 30°, with different amounts of $Na_2S_2O_3$. KOH (0·2 ml. of 10 %) was present in the centre well of each flask. Sulphate was estimated in flask contents at end of reaction.

The molar ratio $Na_2S_2O_3 : O_2 : SO_4^=$ was 1:1·94:2·10.

$Na_2S_2O_3$ added (μ mole)	O_2 absorbed (μ mole)	SO_4 produced (μ mole)
0	0·54	0
4	8·08	7·9
6	11·5	12·7
8	15·8	17·1
10	20·0	20·6

The fixation of carbon dioxide during thiocyanate oxidation

The theory was considered, and is discussed later, that carbon dioxide entered into the oxidative metabolism of thiocyanate. Attempts were made to test the theory by seeking suitable inhibitors that might prevent the fixation of carbon dioxide; the theoretical uptake of oxygen, 2 mole O_2 : 1 mole KCNS, might then be found. Three compounds which have been used for the inhibition of assimilation of carbon compounds by heterotrophs were tested. At a concentration known to inhibit heterotrophic assimilation sodium azide completely inhibited oxidation of thiocyanate. When the concentration was reduced from M/100 to M/1000 the inhibition was still 85–90 % and the compound was therefore unsuitable for use with this organism. 2 : 4-Dinitrophenol was employed at different concentrations; even when the concentration was higher than is commonly used for inhibiting heterotrophic assimilation, and the oxidation was partly inhibited, there was still no evidence that fixation of carbon dioxide was decreased. Iodoacetate was tested with similar results (see Table 5). The gas volumes quoted were calculated as oxygen uptakes but, when no KOH was present, the gas volume change represented the resultant of oxygen absorption, carbon dioxide fixation and carbon dioxide production. If inhibition of fixation had occurred the difference in gas volume change measured in the presence and absence of KOH, would increase, due to the greater output of carbon dioxide. Iodoacetate has been described as inhibiting the fixation of carbon dioxide by *Thiobacillus thiooxidans* (Vogler, 1942).

Table 5. *Oxidation of thiocyanate in the presence of* 2:4-*dinitrophenol and iodoacetate*

Washed suspensions of *Thiobacillus thiocyanoxidans*, grown on thiocyanate and suspended in M/15 K Na phosphate buffer (pH 7), were shaken in air at 30°. The concentration of thiocyanate added to the flasks was constant but the inhibitor concentrations were different. At each concentration the reaction was observed in the presence and absence of KOH (0·2 ml., 10 %). The difference in gas volume taken up was calculated as if oxygen only were involved (see text).

Inhibitor	Concentration (M)	Difference in gas uptake (μl.)
2:4-Dinitrophenol	0	80
2:4-Dinitrophenol	1×10^{-4}	96
2:4-Dinitrophenol	5×10^{-4}	83
2:4-Dinitrophenol	$7{\cdot}5\times10^{-4}$	98
Iodoacetate	0	106
Iodoacetate	1×10^{-4}	69
Iodoacetate	1×10^{-5}	128

The simultaneous metabolism of thiosulphate and cyanate

The theory that the simultaneous fixation of carbon dioxide is responsible for the low O_2 : KCNS ratio observed in the presence of KOH is discussed later. It was considered that a similar low ratio might be observed during the oxidation of thiosulphate if carbon dioxide were similarly fixed. It was found that oxygen uptake during oxidation of thiosulphate was the same in the presence as in the absence of KOH and when ammonium sulphate was present as a nitrogen source. However, in metabolizing thiocyanate, the organism actively produced carbon dioxide and ammonia during the oxidation. The simultaneous metabolism of thiosulphate and cyanate was, therefore, examined in an attempt to make the experimental conditions more like those of thiocyanate metabolism. The metabolism of thiosulphate and cyanate was compared with that of each compound alone in an attempt to show whether carbon dioxide was fixed and whether the fixation of carbon dioxide resulted in a lower O_2 : $Na_2S_2O_3$ molar ratio.

The results of these experiments were not altogether satisfactory but appeared to indicate that the molar ratio for the oxidation of thiosulphate was unchanged by the simultaneous cyanate hydrolysis. The fixation of carbon dioxide from cyanate during oxidation of thiosulphate was not reproducible and the highest molar ratio CO_2 fixed : $Na_2S_2O_3$ oxidized was 0·2 : 1 which was only 50 % of that fixed during thiocyanate oxidation. The results were, therefore, of little value in attempting to elucidate the role of carbon dioxide.

The fixation of carbon dioxide from the gaseous phase after oxidation of thiosulphate was determined by releasing carbon dioxide in the closed system. Corrections were made for the gas which went into solution. Again the results were not reproducible. The maximum ratio CO_2 fixed : $Na_2S_2O_3$ oxidized was 0·12 mole : 1 mole. This suggested that the fixation of carbon dioxide was even less efficient when it did not occur simultaneously with the oxidation of the sulphur compound.

The influence of growth substrate on oxidation rates

A comparison was made of the rates of oxidation of thiocyanate and thiosulphate by cells which had been grown on thiosulphate or on thiocyanate. As the suspensions from thiosulphate media contained a considerable amount of particulate sulphur which was not removed in washing the two suspensions could not be adjusted by turbidity for comparison. The suspensions were prepared making a rough allowance for the sulphur content, and total nitrogen values were determined in order to provide a basis of comparison. The nitrogen concentrations were 150 μg. N/ml. for the suspension grown on thiocyanate and 140 μg. N/ml. for the suspension grown with thiosulphate. Table 6 shows the Q_{O_2}(N) values. The values quoted for thiocyanate and thiosulphate oxidation have been corrected for the endogenous respiration. The high 'endogenous respiration' of the cells grown on thiosulphate might have been due to the oxidation of sulphur. The organisms will grow on elementary sulphur (Happold *et al.* 1954), but oxidation of elementary sulphur has not been demonstrated in manometric experiments. The rate of oxidation of thiocyanate by cells grown on thiosulphate increased with time.

Table 6. *A comparison of the rates of thiocyanate and thiosulphate oxidation by cells grown on thiosulphate of thiocyanate*

Washed suspensions of *Thiobacillus thiocyanoxidans*, grown on thiocyanate or thiosulphate, and suspended in M/15 K Na phosphate buffer (pH 7) were shaken in air at 30°. KOH (0·2 ml., 10 %) was present in all flasks. Values quoted for thiocyanate and thiosulphate oxidation have been corrected for the endogenous rate.

Growth substrate	Respiration substrate	Q_{O_2}(N)
KCNS	None	10
KCNS	KCNS	1363
KCNS	$Na_2S_2O_3$	983
$Na_2S_2O_3$	None	121
$Na_2S_2O_3$	KCNS	100
$Na_2S_2O_3$	$Na_2S_2O_3$	678

The oxidation of thioacetamide and thioacetate

Cells which had been grown on thiocyanate oxidized thioacetamide ($CH_3.CS.NH_2$) to sulphate but at a slower rate than thiocyanate. Table 7 shows the oxygen absorption and sulphate production with thioacetamide. A large endogenous respiration occurred in this experiment because a heavy cell suspension was employed. In order to determine whether the organism was directly metabolizing thioacetamide, a comparison was made between the anaerobic metabolism of thiocyanate and that of thioacetamide. No sulphide was produced from thioacetamide. It was also observed that the rate of oxidation of thioacetamide was not increased when cell suspensions of greater density were employed. Potassium thioacetate ($CH_3.CSK$) was less stable in solution and sulphide was detectable within a short time of preparation. The rate of oxidation of thioacetate was comparable to the rate of oxidation of thiocyanate.

Table 7. *The oxidation of thioacetamide*

Washed suspensions of *Thiobacillus thiocyanoxidans* grown on thiocyanate and suspended in M/15 KNa phosphate buffer (pH 7) were shaken in manometer flasks in air at 30°, with different amounts of thioacetamide. KOH (0·2 ml., 10 %) was present in all flasks. Sulphate was estimated in the flask contents at the end of the reaction.

The molar ratio, $CH_3CSNH_2:O_2:SO_4$ estimated graphically (see text) was 1:2·06:0·93.

Thioacetamide added (μ mole)	Oxygen absorbed (μ mole)	SO_4 produced (μ mole)
0	3·84	0·44
1·6	7·14	1·50
2·4	8·93	2·0
3·2	10·67	2·97
4·0	12·01	4·28

The oxidation of other sulphur compounds, formaldehyde and formate

The following compounds were not oxidized: potassium dithionate, potassium trithionate, potassium sulphate, potassium pyrosulphate, thiourea, cysteine. The possibility that some oxidation occurred with sodium sulphite, sodium tetrathionate, potassium metabisulphite and sodium dithionite was not eliminated. The difficulty in interpreting results with these compounds was due to their instability and the small observed differences between the rate of oxygen uptake by living and heat-killed suspensions on these substrates.

In the equation which has been suggested for the metabolism of thiocyanate the fixed carbon appears as (CH_2O). A test was made with chromotropic acid for formaldehyde after thiocyanate oxidation. It was thought that, if the later stages of autotrophic carbon fixation resembled those of heterotrophs, intermediates might accumulate in the presence of 2:4-dinitrophenol. No formaldehyde was found in these conditions. The reaction has not been carried out in the presence of a fixative for formaldehyde. Washed suspensions of *Thiobacillus thiocyanoxidans* oxidized formate.

DISCUSSION

Thiobacillus thiocyanoxidans hydrolyses thiocyanate to sulphide and it is reasonable to suppose that the other product of hydrolysis is cyanate, since this compound is hydrolysed by the organism to carbon dioxide and ammonia. The equations which represent these steps are:

$$KCNS + H_2O = KSH + HCNO;$$

$$HCNO + H_2O = NH_3 + CO_2.$$

Sulphide was oxidized quantitatively to sulphate and the molar ratio $O_2 : Na_2S$ found of 1·7 : 1 approaches the ratio required by the equation: $Na_2S + 2O_2 = Na_2SO_4$. The quantitative production of sulphate found indicated that the experimental precautions were successful in preventing loss of sulphide from the system, and the low oxygen ratios might be explained as the result of distillation of sulphide into the cell suspensions during equilibration.

The molar ratio for the oxidation of thioacetamide, $O_2 : CH_3CSNH_2 = 2{\cdot}06 : 1$, may also supply evidence for the oxidation of sulphide. It is probable that the normal equilibrium in solution does not favour the production of sulphide, but that the constant removal of traces of sulphide by the organism produces a shift in the equilibrium. The organism did not split off sulphide from thioacetamide as it did in the case of thiocyanate. Since the rate determining step was the slow hydrolysis of thioacetamide, the rate of oxidation was not increased by increasing the cell concentration. The different rate of oxidation of thioacetate, which hydrolyses rapidly in solution, supported this theory. The oxidation of thioacetamide is, therefore, considered to show the quantitative oxidation of sulphide under conditions where the loss of sulphide was decreased to a minimum.

The ability of cells grown on thiocyanate to oxidize thiosulphate while cells grown on thiosulphate showed little ability to metabolize thiocyanate, suggested that thiosulphate might be an intermediate in thiocyanate oxidation, but this was not directly demonstrated. An alternative scheme for the oxidation of sulphide was through sulphite, but the rate of oxidation of sulphite in the presence of living cells was so little greater than the rate in the presence of killed cells that it appeared unlikely that sulphite was an intermediate. Similarly, no evidence was found for the oxidation of thiosulphate through polythionates. The following scheme is therefore suggested:

$$
\begin{aligned}
&(1)\quad 2KSH + 2O_2 = K_2S_2O_2 + 2OH^-,\\
&(2)\quad K_2S_2O_2 + 2OH^- + H_2O = K_2S_2O_3 + 2H_2O,\\
&(3)\quad K_2S_2O_3 + O_2 = K_2S_2O_5,\\
&(4)\quad K_2S_2O_5 + O_2 = K_2S_2O_7,\\
&(5)\quad K_2S_2O_7 + H_2O = K_2SO_4 + H_2SO_4.
\end{aligned}
$$

Thiosulphurous acid, $H_2S_2O_2$, only exists as esters, and these on hydrolysis in the presence of alkali yield thiosulphate. It is possible that step 2 occurs spontaneously. Pyrosulphate does not exist in solution but goes to sulphate as indicated by step 5. The compound $K_2S_2O_5$ does not exist as such in solution so that the uncertain results obtained on the addition of metabisulphite to cell suspensions could neither confirm nor disprove this theory. The overall reaction for this scheme was the theoretical equation for sulphide oxidation: $2KSH + 4O_2 = K_2SO_4 + H_2SO_4$. The oxidation of thiosulphate followed the theoretical equation given previously. This scheme, although not proved, is in agreement with the experimental data.

It will be seen that the hydrolysis of thiocyanate and the oxidation of sulphide, excluding the process of carbon dioxide fixation, occur according to the equation proposed by Happold & Key (1934): $2KCNS + 4H_2O + 4O_2 = K_2SO_4 + (NH_4)_2SO_4 + 2CO_2$. The amounts of ammonia and sulphate formed from thiocyanate were in agreement with this equation, but the oxygen absorption and carbon dioxide production were not. Since the production of the theoretical amount of sulphate was observed it appeared that some other compound was serving as source of oxygen, either directly or indirectly. In the system the possible sources of oxygen appeared to be carbon dioxide and water. It was thought that carbon dioxide was the most likely source since the fixation of carbon dioxide necessitates its reduction. If oxygen had been derived from water, hydrogen should have been produced; there did not appear to be any likely hydrogen acceptors present other than oxygen and carbon dioxide and the production of free hydrogen should have led to lower gas uptakes than were observed.

The difference between the theoretical amount of CO_2 to be expected and that found experimentally was equivalent to the difference between the theoretical and observed O_2 uptakes. It was possible to envisage the reaction of carbon dioxide with a sulphur compound at some stage, but the following mechanism appeared to be more satisfactory: $CO_2 + H_2O = CH_2O + O_2$. The production of one molecule of oxygen for each molecule of carbon dioxide fixed was sufficient to explain the apparent deficiency in the oxygen absorbed. The combination of this equation with the equations for the hydrolysis of KCNS and the oxidation of sulphide yields the overall equation which was suggested by the experimental results, i.e. $2KCNS + 5H_2O + 3O_2 = K_2SO_4 + (NH_4)_2SO_4 + CO_2 + (CH_2O)$.

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Exocellular Chitinase from a *Streptomyces* sp.

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SUMMARY: A number of cultures of chitin-decomposing aerobic bacteria were isolated by the enrichment technique. Chitin decomposition was followed by measurement of loss of alkali-resistant insoluble substrate. Agitated submerged cultures secured a much more rapid breakdown of chitin than static cultures. The exocellular chitinase produced by a species of *Streptomyces* in agitated submerged culture was studied. The effects of pH value and substrate concentration on enzyme activity were examined and the results used to establish a chitinase assay in terms of reducing-sugar production. The enzyme was an inducible one (adaptive). The enzyme was concentrated by ultrafiltration and lyophilization. The water-soluble reducing materials produced by the enzyme from chitin were identified as *N*-acetylglucosamine and its corresponding disaccharide, *N*.*N*-diacetylchitobiose.

Chitin is a tough, pliable, widely distributed natural product resembling cellulose; its fibrous structure is derived from long unbranched chains of *N*-acetylglucosamine units connected by β-1,4-glucosidic linkages. Chitin is extremely resistant to chemical reagents; strong mineral acids are required to dissolve it.

Most of the published reports of microbial chitin decomposition are concerned with taxonomic and physiological studies rather than with the enzymic aspects of the process. Benecke (1905), the first worker to isolate and study a chitin-decomposing microbe, stated that his '*Bacillus chitinovorus*' (a non-sporeformer) must possess a chitinase, but found no evidence of saccharidic products of chitinase activity. Folpmers (1921) suggested the existence of a water-soluble cell-free chitinase from the fact that his cultures of chitin-decomposing Eubacteriales and actinomycetes produced clear zones in agar prepared with precipitated chitin. ZoBell & Rittenberg (1938), from experiments with marine chitin-decomposing bacteria, suggested that chitin breakdown might result from either a cleavage of the glycosidic linkages or a splitting-off of the aminoacetyl group.

Karrer & Hofmann (1929) used a snail gut enzyme to secure up to 85% conversion of chitin into *N*-acetylglucosamine, thus helping to establish that compound as the unit building block of the polysaccharide. No other enzymic breakdown products of chitin were identified. Grassmann & Rubenbauer (1931) found that 'Luizym', a commercial product prepared from dried aqueous extracts of *Aspergillus niger*, split chitin into iodometrically detected reducing material of undetermined nature. Zechmeister, Tóth & Balint (1938) and Zechmeister & Tóth (1939) demonstrated that almond emulsin could be chromatographically separated into a β-glucosidase, an α-galactosidase and a chitinase. The latter was further separated into a polysaccharidase and a second enzyme which preferentially hydrolysed the disaccharide building block of chitin, *N.N*-diacetylchitobiose. Jeuniaux (1950, 1951) found that chitin-decomposing Eubacteriales from the snail gut and chitinoclastic soil actinomycetes produced exocellular chitinases.

EXPERIMENTAL

Preparation of chitin substrate

To secure a substrate for culture media, a supply of partly purified chitin was prepared according to the usual acid and alkali method, with the realization that a certain amount of denaturation was unavoidable. Shell fragments (<2 mm.) of the Dungeness crab, *Cancer magister*, were held in a mechanically stirred suspension of dilute HCl and maintained at pH 2·0–3·0 by repeated addition of acid for 3 days to remove calcium salts. After washing, the material was suspended in 4 vol. of 5 % (w/v) KOH and brought to 100° in an autoclave for 3 hr., with intermittent mechanical stirring, to render the non-chitinous constituents soluble. The alkali treatment was repeated, the chitin washed and then dried at 70°. After grinding through a 0·5 mm. mesh screen Wiley mill, the acid and alkali treatments were repeated, the oven-dried chitin Soxhlet extracted with 95 % ethanol, and the dry powder ground in a ball mill. The yield was 17 %.

A purified lot of chitin for use as a substrate for the assay of chitinase was prepared by extracting the above material with 2·5 % (v/v) acetic acid to remove any chitosan possibly formed by the KOH treatment, followed by 95 % ethanol extraction. The product was almost white in colour and had an ash content of 1·25 %. Microchemical analysis gave the following results: calculated—C, 47·3; H, 6·4; N, 6·89; —$COCH_3$, 22·17 %; found—C, 43·1; H, 7·0; N, 6·87; —$COCH_3$, 19·2 %.

Colloidal precipitated chitin was prepared roughly according to the procedure of Bucherer (1935) and preserved in the frozen state.

Isolation and testing of chitin-decomposing bacteria

Enrichment cultures were set up using a mineral salt medium (K_2HPO_4, 0·7 g.; KH_2PO_4, 0·3 g.; $MgSO_4.5H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; $ZnSO_4$, 0·001 g.; distilled H_2O, 1 l.; pH adjusted to 7·0) to which powdered chitin (0·25 %, w/v) was added as a sole source of C and N. The medium was dispensed in 5 mm. layers in Erlenmeyer flasks; separate flasks of each medium were inoculated with dilutions of eight garden and forest soils. The flasks were incubated both statically and on a rotary table shaker (New Brunswick Scientific Co.) which described a circle of 2·5 cm. diam. and ran at 325 r.p.m. All culture incubation described in this paper was at 28°.

To isolate pure cultures of chitin-decomposing microbes, we plated out the above enrichments after 2 weeks on to neutral mineral salts agar containing sufficient colloidal precipitated chitin to render it cloudy. Bacteria were isolated and purified from 41 colonies which formed clear zones in the chitin. The above enrichment procedure, using chitin as a sole C and N source, admittedly selected against nutritionally fastidious chitin-decomposing microbes.

These 41 isolates were tested for ability to decompose chitin in agitated submerged culture, following approximately the method of Reese (1947) for aerobic cellulose decomposition studies. Portions (250 mg.) of powdered chitin were weighed into 250 ml. Erlenmeyer flasks which were uniformly inoculated and incubated on the rotary shaker for 15 days. Sufficient 5 N-KOH was then added to make the KOH concentration 0·5 N, all growth submerged in the alkaline solution, and the flasks autoclaved at 121° for 15 min. The alkali-insoluble residue was collected in a tared 30 ml. Selas semi-porous crucible (No. 3001), washed with water, and dried at 105° for 16 hr. The cooled crucibles were weighed, ignited for 8 hr. at 650°, and reweighed for ash. An experiment with uninoculated flasks showed that $100 \pm 1\,\%$ of the chitin was recovered by this method. The decomposition of organic matter was calculated as

$$\%\text{ loss of organic matter} = \frac{[\text{post-alkali residue}] - [\text{culture ash}]}{[\text{original chitin, water-free}] - [\text{culture ash}]} \times 100.$$

Since the bacteria formed a certain amount of alkali-resistant polysaccharide during growth, the actual percentage of chitin loss was always somewhat greater than indicated by the data.

When subjected to the above screening procedure, all of the zone-producing organisms showed some ability to decompose chitin in aerated submerged culture. The amount of chitin decomposed ranged between 10 and 97 % of the starting material. In a number of cases, the amount of decomposition strikingly increased when yeast extract (0·05 %, w/v) was added to the medium, e.g. in one instance from 17 to 90 % chitin loss over 15 days. The study of such nutritional aspects of chitin decomposition will be reported later. For the purpose of this investigation, in which a culture fluid with chitinase activity was sought, those cultures which could actively decompose chitin with that compound as a sole source of C and N were chosen for further study. The avoidance of complex organic media would render subsequent enzyme concentration and purification less difficult.

The speed of chitin breakdown with the three most active organisms in the above experiment (two *Streptomyces* spp. and a true bacterium) was ascertained by inoculating each organism into eight replicate flasks of the same medium, and removing pairs at intervals for alkali treatment. The results (Fig. 1) showed that a rapid chitin breakdown took place in submerged cultures of all three organisms. With static incubation of 5 mm. deep layers of media, the amount of chitin breakdown after a month was only a small fraction of that secured with shaken flasks of these aerobic cultures in 5 days.

Demonstration of a cell-free microbial chitinase

The above data were used as a guide to harvesting identical submerged cultures of the three organisms for an examination for chitinase activity in the culture fluids. After 4 days' growth with the *Streptomyces* spp. (C-10 and C-14) and 8 days for the bacterium (C-25), the cell material was centrifuged off and the supernatants preserved by the addition of sufficient 1 % (w/v) aqueous thiomersalate to produce a concentration of 0·01 % (w/v). The three solutions were each divided into equal parts which were adjusted, respectively, to pH's 5·0 and 8·0.

A test for chitinase activity was made on these solutions by dispensing 50 mg. of the ball-milled highly purified chitin into 50 ml. Erlenmeyer flasks, adding 10 ml. of the cell-free fluid so as to produce a homogeneous suspension, and incubating the tinfoil-capped flasks for 48 hr. at 28° on a slower (120 r.p.m.) table shaker running in a 2·5 cm. diam. circle. Two controls were used: (i) flasks with the chitin in mineral salt solution; (ii) cell-free culture fluid without chitin; both controls contained thiomersalate. After incubation, the enzyme was inactivated by immersing the flasks for 5 min. in a 100° water-bath. Approximately 3 ml. of the flask suspensions were centrifuged at 18,000 *g* to remove residual chitin. Samples (1·0 ml.) of the supernatant solutions were added to pairs of optically uniform Pyrex test tubes for reducing-sugar assay by the dinitrosalicylic acid (DNS) method of Sumner & Somers (1949) with the assay adjusted to the range 0·05–0·9 mg. glucose/ml. While both thiomersalate and phosphate salts can interfere with the DNS assay, this was not observed with the concentration of these substances present in the culture supernatants.

Table 1 summarizes the results of the above test at pH 8·0 for chitinase activity in the three organisms. The reducing-sugar found in flasks containing cell-free culture fluid was appreciably higher than that found in flasks containing fluid alone. In this and all subsequent experiments, the control flasks with cell-free fluid alone contained either no or at most a trace of reducing substance detectable by the procedure used. Likewise, the controls with chitin incubated with mineral salts solution were consistently negative for reducing sugars. *Streptomyces* sp. C-10 was selected for further enzyme work.

Table 1. *Chitinase activity of cell-free culture fluids from three submerged cultures of chitin-decomposing bacteria*

The figures represent averages of two flasks, each assayed in duplicate.

Culture	Solution assayed	Reducing-sugar as glucose (mg./ml.)
C-10	Cell-free culture fluid	<0·05
	Cell-free culture fluid+chitin	0·38
C-14	Cell-free culture fluid	<0·05
	Cell-free culture fluid+chitin	0·26
C-25	Cell-free culture fluid	0·06
	Cell-free culture fluid+chitin	0·15
None	Chitin+min. salt solution	<0·05

The observed chitinase activity was shown to be due to a cell-free enzyme (or enzymes) by filter sterilization of active centrifuged C-10 culture fluid with Morton Ultra Fine porosity sintered glass filters. Solutions so sterilized had a chitinase activity; a sterility test showed that no contamination took place before or during the period of enzyme activity.

Some properties of the enzyme

pH *range.* With thiomersalate-treated cell-free culture fluid, enzyme solutions containing M/15 phosphate were prepared over the pH range 4·5–8·3; the range was extended to pH 3·0 with HCl. Additional buffer capacity to that of the phosphate was presumably provided by dissolved protein. Duplicate 10 ml. samples were assayed as above in 50 ml. flasks containing 30 mg. chitin incubated on the shaker at 28° for 72 hr. The pH change in all but three of the nineteen different values of enzyme solution did not exceed 0·2 unit during the experiment; in three, it did not exceed 0·4 unit. The assay results (Fig. 2) indicated a pH optimum *c.* pH 6·5–7·5, so that subsequent assays were run at pH 7·0.

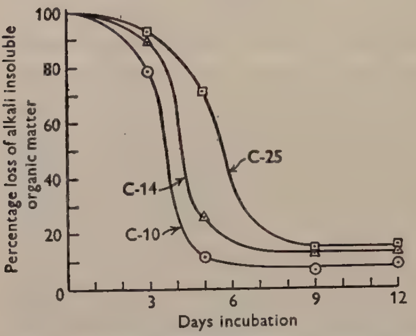

Fig. 1. Aerobic decomposition of chitin by three soil organisms in agitated submerged culture; C-10 and C-14 *Streptomyces* spp., C-25 a bacterium.

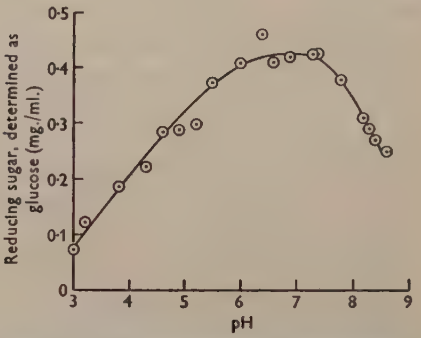

Fig. 2. The effect of pH on the activity of *Streptomyces* sp. C-10 chitinase.

Effect of substrate concentration. Chitin was distributed into pairs of Erlenmeyer flasks (75 mg./flask) and cell-free culture fluid added as follows: 10 ml./50 ml. flask; 25 ml./125 ml. flask; 50 ml./250 ml. flask; 75 ml./300 ml. flask. The flasks thus containing 7·5, 3·0, 1·5 and 1·0 mg. chitin/ml. solution were then assayed with controls, using 28° and 48 hr. incubation. In all the flasks, a large portion of the insoluble chitin was still visible after the assay. The results of the experiment (Fig. 3) led to the selection of 3 mg. chitin/ml. (actually 30 mg./10 ml.) for all subsequent assays. Since chitin is insoluble in water, one can readily establish that this substrate has not become exhausted during the course of a chitinase assay. Kinetic experiments have also shown that 3·0 mg. chitin/ml. is an adequate substrate concentration for assay work.

Improvements in the assay were later made when it was found that the enzyme had a temperature optimum at 37°. While at 20 and 28° rotary agitation increased the amount of reducing-sugar production, at 37° equal amounts of product were formed in both swirled and static flasks, thus rendering agitation unnecessary.

Enzyme induction by chitin. Streptomyces sp. C-10 was grown in the neutral mineral salt medium in agitated submerged culture with chitin (0·5 %, w/v), *N*-acetylglucosamine (0·3 %, w/v), or glucose (0·1 %, w/v) + asparagine (0·05 %, w/v). The *N*-acetylglucosamine was sterilized by filtration. With the two monosaccharide media, growth of the actinomycete was moderate in amount; with the chitin medium, heavy. After given periods of time, the supernatants from pairs of duplicate flasks were assayed for chitinase activity. The results (Table 2) showed that only with the organisms grown in chitin was there evidence of formation of reducing-sugars from chitin by the cell-free culture fluids; the enzyme thus exhibited an induced (adaptive) behaviour.

Two β-1,4-linked polymers of glucose configuration sugars (cellulose and cellobiose) were not hydrolysed by the enzyme; it appears that if compounds of this type are to be cleaved by the enzyme, they must possess the amino-acetyl group.

Table 2. *Effect of substrate on chitinase production by* Streptomyces *sp.* C-10

Compounds added to basal mineral salt medium	Age of culture (days)	Enzyme activity of cell-free culture fluid		Reducing-sugar, as glucose, produced by chitinase (mg./ml.)
		Alone	With chitin	
		Reducing-sugar formed, as glucose (mg./ml.)		
		(1)	(2)	(2) − (1)
Glucose + Asparagine	5	0·77	0·78	0·1
	6	0·35	0·35	0·0
N-acetylglucosamine	3	0·95	0·95	0·0
	5	<0·05	<0·05	0·0
Chitin	5	<0·05	0·28	0·28

Stability of the enzyme. C-10 chitinase activity is very stable when the solutions are treated with 0·01 % (w/v) thiomersalate, brought to pH 7 and stored at 4°. A sample of cell-free fluid stored under these conditions for 4 months showed only about 25 % loss of activity.

Production of the enzyme in mass culture. To determine when maximum chitinase activity was reached, 2 l. Erlenmeyer flasks containing 1 l. of medium were inoculated at intervals over a period of 2 weeks with 50 ml. of a 3-day-old submerged C-10 culture and incubated on the shaker at 250 r.p.m. The flasks, ranging in age from 2 to 14 days, were simultaneously harvested and the culture supernatants assayed in the usual way. The results (Fig. 4) showed that peak chitinase activity was reached at 6 days incubation and fell off thereafter. The peak of activity coincided approximately with the time at which the last remaining chitin disappeared from the bottom of the flask.

A similar time study on chitinase formation was conducted with cultures in which the chitin neutral mineral salts medium received separately different nitrogenous supplements: Difco yeast extract (0·2 or 0·05 %), asparagine (0·1 %), or a mixture of $NaNO_3$ and NH_4NO_3 (0·07 and 0·03 %, w/v). The basal chitin mineral salt medium gave higher peaks of chitinase activity than any of the media which received these nitrogenous supplements. Earlier experiments on chitin break-down by organisms which could grow with chitin as a sole source of carbon and nitrogen had shown that a number of these bacteria were much more effective in decomposing the polysaccharide when yeast extract was added to the medium, but *Streptomyces* sp. C-10 did not respond in this manner to yeast extract supplementation. It was therefore not surprising that this organism produced no increase in exocellular chitinase when yeast extract was added.

Concentration of the enzyme by ultrafiltration and lyophilization

A preliminary concentration of enzyme was made by means of nitrocellulose ultrafilters prepared after the procedure of Kabat & Mayer (1948); a glass mantle surrounding the filter thimble allowed a greater degree of concentration than in their technique. Active culture fluid (10 l.) was chilled to 5°, clarified with a Sharples supercentrifuge, treated with thiomersalate, adjusted to pH 7·0 and concentrated to 100 ml. (100-fold) with an ultrafilter prepared with a 5 % (w/v) cellulose nitrate solution. Controlled chitinase assays on the original solution (0·32 mg. reducing-sugar/ml.), the solution passed by the ultrafilter (< 0·05 mg./ml.) and the solution retained by the ultrafilter (14·0 mg./ml.) indicated that a marked concentration of the enzyme was secured by ultrafiltration. Further concentration of the ultrafilter-retained solution to the dry state without any loss of activity was made by freeze-drying.

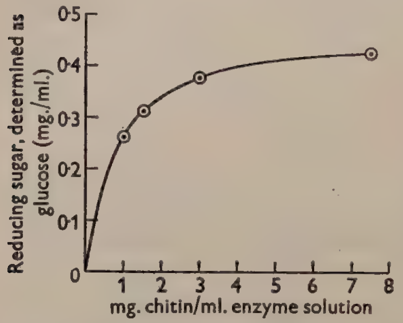

Fig. 3. The effect of substrate concentration on reducing sugar production by *Streptomyces* sp. C-10 chitinase.

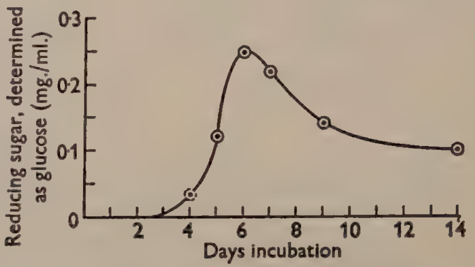

Fig. 4. Production of exocellular chitinase by *Streptomyces* sp. C-10 in agitated submerged culture with a chitin-mineral salts medium at different times of growth.

A brown precipitate appearing on the surface of the ultrafilters was enzymically inactive and was subsequently separated from all ultrafilter concentrates by the Sorvall SS-1 centrifuge at 18,000 *g*.

Identity of chromatographically detectable enzyme products

For chromatographic detection of reducing sugars, the phenylenediamine spray reagent of Chargaff, Levine & Green (1948), modified by Mr F. Stadtman (1953, personal communication) by the substitution of oxalic acid for HCl was used; the reagent is prepared by dissolving 0·2 % (w/v) oxalic acid in approximately 95 % ethanol. *o*-Phenylenediamine (instead of the *meta* isomer used by Chargaff *et al.*) was used, since the former on heating at 100° for 15 min. gave more distinguishable colours in daylight. A preliminary experiment showed that *N*-acetylglucosamine was completely hydrolysed to glucosamine when it was dissolved at 5 % (w/v) in N-HCl and heated to 100° for 5 hr. in a sealed test tube.

A solution of the unknown reducing material produced by the enzyme was obtained by allowing 50 ml. active cell-free culture fluid to react with 500 mg. of highly purified ball-milled chitin in a 500 ml. Erlenmeyer flask at 28° on the 120 r.p.m. shaker. After 7 days, a considerable amount of insoluble chitin remained in the flask. The suspension was heat inactivated, centrifuged and the supernatant passed through a small de-ionizing column filled with a mixture of cationic and anionic exchange resins. The solution was then concentrated to about 0·5 ml. (100-fold) by evaporation *in vacuo* at 70°. A similar control concentrate was made of an identical enzyme solution which had not accumulated reducing material by incubation with chitin. Whatman No. 1 filter-paper was set up with 10 μl. spots of the following materials: glucose (1 %, w/v), glucosamine : HCl (5 %, w/v), *N*-acetylglucosamine, both untreated and acid-hydrolysed (5 %, w/v), the above enzyme concentrates, both control and chitin-reacted, and the latter concentrate acid-hydrolysed as above. Development of the descending 36 hr. phenol/water/ammonia chromatogram showed that the control enzyme preparation contained no detectable reducing compounds, whereas the concentrate of the enzyme previously reacted with chitin showed two spots, one with the same R_F and colour as secured with *N*-acetylglucosamine and a second more rapidly moving unidentified compound. The identity of the slower enzyme product with *N*-acetylglucosamine was confirmed by the fact that the HCl-hydrolysed enzyme products gave a single spot with the same R_F and colour as glucosamine, which is under these conditions the hydrolysis product of *N*-acetylglucosamine.

Since the faster spot was also converted to glucosamine on acid hydrolysis, it seemed likely that it might be *N*.*N*-diacetylchitobiose. We secured 0·75 g. of crystalline octa-acetylchitobiose which had been prepared from chitin after Bergmann, Zervas & Silberkweit (1931). It had an m.p. of 306–308° (decomp.) and $[\alpha]^{23}_{\mathrm{D}} + 56{\cdot}5^{\circ} \pm 0{\cdot}4$ (1 %, HOAc); these values are not in accord with those (m.p. 289° and $[\alpha]^{23}_{\mathrm{D}} + 50{\cdot}3^{\circ}$) secured by Bergmann *et al.*, but are in agreement with those obtained for the same octa-acetate by Zechmeister & Tóth (1931): 305° and $[\alpha]^{23}_{\mathrm{D}} + 55{\cdot}3^{\circ}$. The O-acetyl groups were selectively hydrolysed from the octa-acetate by means of the Isbell barium methylate procedure described by Bates *et al.* (1942), yielding *N*.*N*-diacetylchitobiose.

The above described phenol/water/ammonia chromatogram was repeated with the addition of *N*.*N*-diacetylchitobiose and chitobiose octa-acetate. Both the R_F values and the colours obtained with spots of the respective substances (Fig. 5) confirmed the earlier experiment and indicated that the previously unidentified enzymic product was *N*.*N*-diacetylchitobiose. The chromatogram was repeated with a collidine/water system with the same net results: two enzymic products were evident, with the R_F values and spot colours of *N*-acetylglucosamine and *N*.*N*-diacetylchitobiose. With collidine/water the biose moved more slowly than the *N*-acetylglucosamine. In none of the chromatograms was there evidence that either glucosamine or glucose were produced from chitin by this enzyme. The product solution was examined with a two-dimensional chromatogram using the solvent system phenol/water/ammonia followed by butanol/ethanol/water/ammonia (45 : 5 : 49 : 1). This experiment revealed no more than the two products already identified.

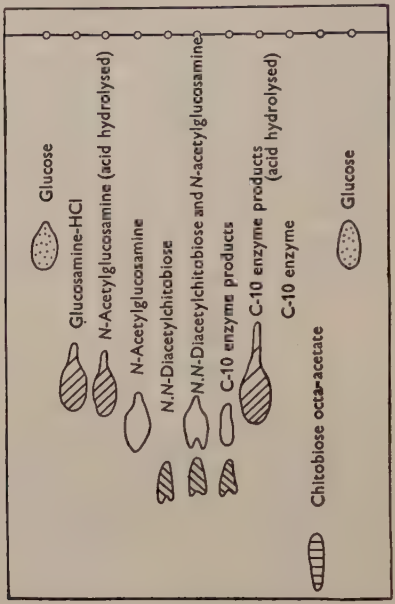
Glucose
Glucosamine-HCl
N-Acetylglucosamine (acid hydrolysed)
N-Acetylglucosamine
N.N-Diacetylchitobiose
N.N-Diacetylchitobiose and N-acetylglucosamine
C-10 enzyme products
C-10 enzyme products (acid hydrolysed)
C-10 enzyme
Chitobiose octa-acetate
Glucose

Fig. 5. Phenol/water/ammonia paper chromatogram of the water-soluble reducing products resulting from the hydrolysis of chitin by *Streptomyces* sp. C-10 chitinase. Spots developed by phenylenediamine and oxalic acid reagent. Colours of spots in daylight: [·]=blue; [/]=brownish green; [ ]=reddish brown; [\ ]= greenish brown; [—]= greenish blue.

DISCUSSION

From our data it appears that enzymic chitin breakdown may follow the pattern of a number of other polysaccharides in that one or more enzymes degrade the compound to a biose stage which is then split by another enzyme. Supporting evidence of this lies in the fact that another similarly prepared chitinase product concentrate on chromatographic examination showed only a *N.N*-diacetylchitobiose spot; no monose was detected. Other polysaccharidases which accumulate bioses include the classical amylose → maltose phenomenon, the cellulose → cellobiose mechanism shown by Levinson, Mandels & Reese (1951) and by Whistler & Smart (1953), the xylan → xylobiose pathway (Sørenson, 1952) and the pectic acid → digalacturonic acid route of Dr A. L. Demain and Dr H. J. Phaff (1954, personal communication).

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Hydrogen Sulphide Production by Non-multiplying Organisms and its Inhibition by Antibiotics

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SUMMARY: Hydrogen sulphide production by washed bacterial suspensions in buffered substrate solutions was examined. The suspensions were tested for H_2S production from cysteine, cystine, homocystine, methionine, mercaptoacetate, sulphite, sulphate or thiosulphate. Some strains of the Proteus, Klebsiella, Salmonella, Arizona and Bethesda groups produced hydrogen sulphide when 10^{11} or more washed organisms/ml. were incubated at 37° without an added source of sulphur. Generally tenfold greater quantities of bacteria were required to produce hydrogen sulphide within 24 hr. from homocystine, sulphite or thiosulphite as compared with cystine or cysteine. For the production of hydrogen sulphide from sodium mercaptoacetate, 10- and 100-fold larger quantities of bacteria were required. In the Brucella group all strains behaved alike, except two non-smooth strains which were spontaneously agglutinated in the buffer + substrate solutions and did not produce hydrogen sulphide. Among the Enterobacteriaceae the most active strains belonged to the Proteus group, the most inactive strains to the Large-Sachs group. All strains of the Arizona and Bethesda groups, and some Klebsiella, Salmonella, Serratia and Providence strains multiplied on cysteine and cystine + buffer solutions, utilizing for growth the split products, pyruvic acid and ammonia.

Hydrogen sulphide production was inhibited by penicillin or streptomycin. Non-multiplying micro-organisms exposed to penicillin, streptomycin, aureomycin or chloramphenicol for 24 hr. at 37° remained viable, but lost their ability to produce hydrogen sulphide. This ability was regained on subculture. The hydrogen sulphide production of bacteria inactivated by penicillin was restored when the cells were removed by centrifugation, and residual penicillin destroyed by penicillinase. Addition of bacterial extracts heated at 50° or pyridoxal phosphate, separately or together to inactivated bacteria, reactivated the hydrogen sulphide production of *Proteus vulgaris*. Bacterial extracts heated at 100° exerted slight reactivating effects.

Hydrogen sulphide production by multiplying and non-multiplying bacteria has been described by many authors (see Clarke, 1953). Mention should be made of some investigations on factors which inhibit or stimulate the production of hydrogen sulphide. Beijerinck (1901) was the first to determine that many micro-organisms, among them escherichias and klebsiellas, were able to produce hydrogen sulphide from sulphite, thiosulphate or elementary sulphur, and that these reactions were more marked under anaerobic conditions. A further observation of Beijerinck (1904) was the 'indirect formation of sulphides' by *Escherichia* and *Klebsiella* spp., i.e. the biosynthesis of sulphur-containing organic compounds from sulphate as S source and the production of sulphides by their decomposition. Braun & Silberstein (1942) reported that hydrogen sulphide production from cystine, thiosulphate or elementary sulphur by Enterobacteriaceae was stimulated by the presence of glucose; the reaction was inhibited by pyruvate. Kallio & Porter (1950) observed the anaerobic breakdown of cysteine to pyruvate, ammonia and hydrogen sulphide by *Proteus vulgaris* and described the reactivation of toluene-treated cells by the addition of boiled cell extract.

Kallio (1951) demonstrated the stimulation of the removal of H_2S from cysteine in *Proteus* sp. by pyridoxal phosphate, and Delwiche (1951) the stimulation of the same reaction in *Escherichia coli* by adenosine-5-phosphate, pyridoxal phosphate, biotin or α-ketoglutarate, and its inhibition by phosphoglyceric acid, serine, alanine or semicarbazide. Özek (1951) found that in the presence of different carbohydrates and alcohols different quantities of hydrogen sulphide were produced by the same strain. Skariton-Loewenthal, Rappaport & Olitzki (1953) found that hydrogen sulphide production by *Salmonella enteritidis* on Kligler's iron agar (Difco) was inhibited by sodium biselenite and penicillin in concentrations which did not damage the vital functions of the bacterial cells.

EXPERIMENTAL

The organisms were grown for 2 days in Roux bottles on nutrient agar, then removed with saline, washed twice by centrifugation and resuspended in saline; 0·5 ml. of the required bacterial suspension was added to 0·5 ml. of the buffer + substrate solution (0·15 % K_2HPO_4; 0·05 % KH_2PO_4; 0·5 % NaCl; substrate). Inorganic substrates were added in a concentration of 0·5 %; organic substrates in a concentration of 0·1 % or, if not soluble at this concentration, at saturation at 37°. The pH value of the mixtures was adjusted to 7·2. All experiments were carried out in 75 × 11 mm. test tubes firmly closed by rubber stoppers after insertion of lead acetate paper. The bacteria + substrate mixtures were incubated at 37° and the beginning of H_2S production noted when blackening of the paper started. Observations were continued for 48 hr., although strongly positive reactions appeared in less than 1 hr.

The formation of soluble sulphides was tested by addition of NaOH and a reagent consisting of 0·1 % lead acetate, 0·15 % acetic acid and 0·25 % gum-arabic (Delwiche, 1951). This reagent occasionally gave weakly positive results when the reaction on lead acetate paper was completely negative. It did not give, however, satisfactory results in the presence of mercaptoacetate since by the mere addition of NaOH, hydrogen sulphide was liberated from this substance. The results were usually first read after 30 min. and then every 2 hr. until 6 hr. from the start of the experiment; 24 and 48 hr. readings were also taken. The velocity of the reaction was graded as follows: 3, positive reaction after a few hours; 2, positive at 24 hr.; 1, positive at 48 hr.; 0, negative reaction at 48 hr.

Another method used to measure relative activity of H_2S production was to dilute the original bacterial suspension containing 10^{11} organisms/ml. in two-fold dilutions and to determine the minimal dilution able to produce a positive reaction within 24 hr. The lowest limit found was 10^8 to 10^9 micro-organisms/ml. This method, however, revealed that certain Enterobacteriaceae were able to grow on cysteine or cystine + buffer solutions even with inocula of only 10 or

100 organisms/ml. Growth and H_2S production were manifested by an increasing turbidity of the medium and a slowly increasing blackening of the lead acetate paper.

In all experiments a control tube was added which contained buffer solution without any sulphur-containing substrate and an inoculum of 10^{11} organisms/ml. This control was necessary since heavy suspensions of certain strains of the Proteus, Salmonella, Klebsiella and paracolon groups evolved hydrogen sulphide even in the absence of an added sulphur-containing compound, by the decomposition of their own sulphur-containing organic substances, as observed by Beijerinck (1904).

RESULTS

Hydrogen sulphide production by Brucella *spp.*

Table 1 shows that smooth strains of all three species of *Brucella* produced hydrogen sulphide from cysteine, cystine, homocystine, mercaptoacetate, Na_2SO_3 and $Na_2S_2O_3$. On homocystine, tenfold greater inocula were required; smooth strains of *B. suis* produced H_2S on this substrate more readily than the other species. On Na_2SO_3 and $Na_2S_2O_3$ the smooth strains of *B. suis* and *B. abortus* produced H_2S more quickly than the smooth strain of *B. melitensis*. On meth-

Table 1. H_2S *production by washed brucellas on six different substrates*

	Cystine and cysteine		Homocystine		Mercapto-acetate		Na_2SO_3 and $Na_2S_2O_3$		
	Size of inocula								
Strains	3×10^{10}	3×10^{9}	3×10^{11}	3×10^{10}	3×10^{10}	3×10^{9}	3×10^{10}	3×10^{9}	3×10^{8}
	Relative H_2S production observed								
Smooth									
***B. melitensis*, 5**	3	1–2	2	0	1	0	2	2	0–1
***B. melitensis*, 7**	3	0–1	2	1	1	0	2	2	1
***B. suis*, 6**	3	2	3	2	2	2	3	2	0
***B. suis*, 39**	3	1–2	3	2	2	1	3	2	0–1
***B. abortus*, 19**	2–3	0	2	0	2	1	3	2	0
***B. abortus*, 2308**	2–3	0	2	0–1	2	1	3	2	0
Non-smooth									
***B. melitensis*, 7**	0	0	0	0	2	0	2	1–2	0
***B. suis*, 6**	2–3	1–2	2	2	2	1	3	2	0–1
***B. abortus*, 2308**	3	1	2	1	2	1	3	1–2	0
Streptomycin resistant									
***B. melitensis*, 5**	3	2	2	2	2	1	3	2	0
B. suis*, 1	0	0	0	0	0	0	0	0	0
B. suis*, 2	3	2	3	0	2	1	3	2	0
B. suis*, 3	2	0–1	2	0	2	1	2	1	0
B. suis*, 4*	2	0	0	0	1	1	2	0	0
***B. abortus*, 19**	3	2	2	1	2	1	3	1	0

3 = strong H_2S production within 6 hr.; 2 = moderate H_2S production within 24 hr.; 1 = weak H_2S production 48 hr.; 0 = after 48 hr. incubation H_2S not produced. * = derived from *B. suis* 6; ** = derived from *B. suis* 39.

ionine only 4 strains, and on Na_2SO_4 only 3 strains out of 15 showed delayed weak reactions after 48 hr. when 3×10^{11} organisms/ml. were used. The non-smooth strains were strongly agglutinated in the buffer + substrate mixtures, particularly those which contained cysteine or homocystine. The non-smooth *B. melitensis* strain did not produce H_2S from these substrates. One of 4 streptomycin-resistant *B. suis* strains (non-smooth) lost completely its desulphhydrase activity for all substrates and another lost its activity for homocystine.

Hydrogen-sulphide production by Enterobacteriaceae

Tables 2 and 3 show that *Proteus* contains the most active H_2S-producing species. All four species (31 strains) produced H_2S from almost all substrates examined, and only relatively small inocula were needed to yield a positive reaction within 24 hr. No Proteus strains tested were able to grow on the substrate + buffer mixture alone.

Table 2. H_2S *production by different species*

Inoculum: 10^{11} organisms/ml.

		No. of strains producing H_2S within 24 hr. on				
Organism	No. of strains examined	Cysteine and cystine	Homo-cystine	Mercapto-acetate	Na_2SO_3	$Na_2S_2O_3$
Proteus vulgaris	15	15	15	7	15	15
P. mirabilis	6	6	6	4	6	6
P. rettgeri	5	5	2	1	5	1
P. morganii	5	5	5	2	5	2
Providence group	6	6	6	6	5	6
Klebsiella spp.	12	12	8	9	12	12
Salmonella spp.	12	12	4	6	9	10
Arizona group	5	5	2	2	5	2
Bethesda-Ballerup	6	6	2	2	3	2
Serratia spp.	2	2	2	0	2	2
Escherichia spp.	10	10	0	0	8	8
Alkalescens-dispar	6	6	6	2	6	6
Shigella flexneri	6	6	0	0	0	2
S. boydii	8	8	3	0	2	3
S. sonnei	3	3	2	0	2	2
S. dysenteriae type 2 (Schmitz)	2	2	1	0	0	1
S. dysenteriae type 1 (Shiga)	5	5	2	0	0	0
Large-Sachs group	4	4	0	0	0	0

Another group of organisms also proved to be very active, but these were able to grow on cysteine or cystine. All strains of the Arizona and Bethesda-Ballerup groups grew when inocula of *c.* 10 organisms/ml. were used; other groups (Providence, Salmonella, Klebsiella, Serratia) contained some strains which were able to grow on cystine or cysteine. Among the salmonellas four organisms grew on cystine or cysteine as sole source of nitrogen, sulphur and energy, namely *Salmonella paratyphi-B*, *S. cholerae-suis*, *S. typhi-murium* and *S. enteriditis*. All these organisms were also able to grow on plain agar plates to which were added only pyruvate and ammonium sulphate. According to

Kallio & Porter, pyruvate, ammonia and H_2S are the enzymic split-products of cysteine. It seems, therefore, that these organisms were able to decompose cystine and cysteine and to use the products for growth. The Proteus strains did not grow in cysteine + buffer solution or in pyruvate + ammonium agar, but they grew when $1{\cdot}0\,\mu g.$ nicotinamide/100 ml. was also added.

Table 3. *Minimal inocula required for hydrogen sulphide formation within 24 hr.*

	Substrate				
Organism	Cysteine and cystine	Homo-cystine	Mercapto-acetate	Na_2SO_3	$Na_2S_2O_3$
	Minimal inocula (cells/ml.)				
Proteus vulgaris	$2{\cdot}5 \times 10^8$	2×10^9	10^{10}	2×10^9	3×10^9
P. mirabilis	$1{\cdot}5 \times 10^8$	5×10^8	10^{10}	2×10^9	4×10^9
P. rettgeri	$2{\cdot}5 \times 10^8$	4×10^9	10^{10}	3×10^9	1×10^{10}
P. morganii	$3{\cdot}5 \times 10^8$	2×10^9	10^{10}	5×10^9	1×10^{10}
Providence	G or $2{\cdot}5 \times 10^8$	4×10^9	10^{10}	1×10^{10}	6×10^9
Klebsiella spp.	G or $2{\cdot}5 \times 10^8$	1×10^{10}	10^{10}	8×10^9	9×10^9
Salmonella spp.	G or $6{\cdot}0 \times 10^9$	1×10^{10}	10^{11}	4×10^{10}	2×10^{10}
Arizona	G	1×10^{10}	10^{10}	1×10^{10}	1×10^{10}
Bethesda-Ballerup	G	6×10^{10}	10^{10}	1×10^{10}	1×10^{10}
Serratia spp.	G or $1{\cdot}0 \times 10^9$	1×10^{10}	N	1×10^{11}	1×10^{10}
Escherichia spp.	$7{\cdot}0 \times 10^8$	N	N	3×10^9	4×10^9
Alkalescens-dispar	$1{\cdot}0 \times 10^9$	1×10^{10}	10^{11}	4×10^{10}	1×10^{10}
Shigella boydii	$4{\cdot}5 \times 10^{10}$	1×10^{11}	N	1×10^{11}	1×10^{11}
S. sonnei	$7{\cdot}5 \times 10^9$	1×10^{11}	N	1×10^{11}	5×10^{10}
S. flexneri	$1{\cdot}0 \times 10^{11}$	N	N	N	1×10^{11}
S. dysenteriae, type 2	$1{\cdot}0 \times 10^9$	1×10^{11}	N	N	1×10^{11}
S. dysenteriae, type 1	$7{\cdot}0 \times 10^9$	1×10^{11}	N	N	N
Large-Sachs	$7{\cdot}5 \times 10^{10}$	N	N	N	N

G = growth on the substrate + buffer solution;
N = H_2S formation not observed after an incubation of 48 hr. with an inoculum of 10^{11} micro-organisms.

It should be mentioned that H_2S production by the bacteria on the substrate + buffer solution was not paralleled by their ability to produce H_2S when grown in Kligler's iron agar (Difco). *Salmonella paratyphi-A* showed almost the same activity on the buffer + substrate solution as did *S. paratyphi-B*, but on Kligler's iron agar only *S. paratyphi-B* yielded a positive H_2S reaction. A similar observation was made with three streptomycin-resistant strains of *S. typhi*: on Kligler's iron agar they lost completely their ability to produce hydrogen sulphide (cf. Seligmann & Wassermann, 1947) but in buffer + cystine, cysteine, mercaptoacetate, sulphite or thiosulphite they were almost as active as their streptomycin-sensitive parent strain.

With the Escherichia and alkalescens-dispar groups and the species *Shigella flexneri*, *S. boydii*, *S. sonnei* and *S. dysenteriae* type 2, relatively large inocula were required to produce H_2S from cystine or cysteine; no strain grew on these compounds, few strains produced H_2S from homocystine (inoculum of 10^{11} organisms/ml.), and only two strains produced H_2S from mercaptoacetate.

The least active organisms were *Shigella dysenteriae* type 1 (Shiga's bacillus) and the strains belonging to the Large-Sachs group. None produced H_2S from sulphite, thiosulphate or mercaptoacetate; two strains formed H_2S from homocystine. For H_2S production from cysteine or cystine *c.* 10^{11} organisms/ml. were required. None of the organisms belonging to the Shigella group produced H_2S on Kligler's iron agar.

Penicillin inhibition of H_2S *formation by non-multiplying cells in the presence of substrate*

Non-multiplying cells from three *Brucella* species were incubated, each with four different substrates and four different concentrations of penicillin; the results of this experiment are summarized in Table 4. In the presence of 20,000 units penicillin/ml. H_2S production from cysteine, cystine, sulphite or thiosulphate was completely inhibited. In the presence of 2000 units penicillin/ml. a marked decrease of H_2S production was observed when smaller inocula were used. This inhibition by penicillin was more marked with cysteine or cystine than with thiosulphate or sulphite.

Table 4. *Inhibition by penicillin of* H_2S *production by brucellas*

			Reaction observed with inoculum of			
Strain	Substrates	Penicillin (u./ml.)	1×10^{10}	5×10^{9}	2×10^{9}	1×10^{9}
B. melitensis	C	None	3	2	2	0–1
	S		3	2	2	2
	C	200	3	2	0–1	0–1
	S		3	2	2	1
	C	2,000	2	0–1	0	0
	S		2	0–1	0	0
	C	20,000	0	0	0	0
	S		0–1	0	0	0
B. suis	C	None	3	2–3	2–3	2
	S		3	2	2	1–2
	C	200	3	2–3	2	2
	S		3	2	2	2
	C	2,000	3	1–2	0–1	0
	S		2	2	2	0–1
	C	20,000	0	0	0	0
	S		0–1	0	0	0
B. abortus	C	None	2–3	1–2	1	0
	S		3	2	2	1–2
	C	200	2	1–2	1–2	0
	S		3	2	2	1–2
	C	2,000	1–2	0	0	0
	S		3	2	1–2	0
	C	20,000	0	0	0	0
	S		0	0	0	0

C = cystine or cysteine; S = Na_2SO_3 or $Na_2S_2O_3$. 3, 2, 1, 0 for relative H_2S production have the same significance as in Table 1.

After incubating two suspensions (10^{10} and 5×10^{9} organisms/ml.) with

20,000 units penicillin/ml. and the above-mentioned substrates for 48 hr. the cells were removed by centrifugation, washed in buffer solution and resuspended in the original substrate + buffer solutions without penicillin. All three organisms (*Brucella melitensis*, *B. suis*, *B. abortus*) with both sizes of inoculum showed H_2S production from cysteine or cystine but not from sulphite or thiosulphate. It seemed, therefore, that some enzyme or enzymes which acted on the latter substrates had been destroyed by penicillin, while the enzymes concerned with cysteine and cystine were only inhibited by penicillin and restored to activity when it was removed. The viability of the cells was not damaged by the exposure to penicillin.

Effect of streptomycin on H_2S *production*

One strain, *Brucella abortus* 2308, was found in which the cysteine desulphhydrase appeared to be more sensitive to streptomycin than the enzymes which produced H_2S from the other substrates tested. The cells were exposed to different concentrations of streptomycin and the required substrate. H_2S production from sulphite or thiosulphate remained active even in the presence of 100,000 μg. streptomycin/ml., but H_2S production from cystine or cysteine ceased in the presence of 1000 μg./ml. (Table 5). The cells were allowed to remain in contact with substrate + 100,000 μg. streptomycin/ml. for 48 hr., then removed by centrifugation, washed in buffer solution and resuspended in the substrate-buffer solution; H_2S was then no longer produced. A similar experiment was carried out with *Salmonella typhi* O901 and three streptomycin-resistant mutants derived from it. The results, summarized in Table 6, showed that washed suspensions of strain O901 and its three streptomycin-resistant mutants produced H_2S from cysteine; on Kligler's iron agar, only the original strain produced H_2S, while the streptomycin-dependent mutants failed to do so. The desulphhydrase activity of all four strains was markedly inhibited by 20,000 μg. streptomycin/ml. When the cells were removed by centrifugation, washed and again resuspended in the cysteine + buffer solution, H_2S production was not restored, while in a control abundant H_2S production occurred.

Table 5. *Inhibition of* H_2S *production by* Brucella abortus 2308 *by streptomycin*

Substrates: cysteine and cystine.

Streptomycin (μg./ml.)	**Inoculum size (cells/ml.)** 10^{10}	5×10^9	2×10^9
	Relative H_2S production		
None	3	3	2
10	3	3	2
100	2	2	2
1,000	1	0	0
10,000	0	0	0
100,000	0	0	0

Table 6. *Inhibition by streptomycin of* H_2S *production by* Salmonella typhi O901 *and three streptomycin-resistant mutants*

Substrate: cysteine.

Streptomycin (µg./ml.)	Strain	Inoculum size (cells/ml.)					
		2×10^{11}	1×10^{11}	5×10^{10}	2×10^{10}	1×10^{10}	5×10^{9}
		Relative H_2S production					
None	O901 R1	3	2	2	1	0	0
	O901 R2	3	2	2	1	0	0
	O901 R3	3	2	2	0	0	0
	O901 Orig.	3	3	3	3	2	1
2,000	O901 R1	2	1	0	0	0	0
	O901 R2	2	1	1	0	0	0
	O901 R3	2	2	0	0	0	0
	O901 Orig.	3	3	3	3	0	0
20,000	O901 R1	1	0	0	0	0	0
	O901 R2	2	1	1	0	0	0
	O901 R3	1	0	0	0	0	0
	O901 Orig.	3	3	2	0	0	0
20,000; after 48 hr. cells washed and resuspended in cysteine buffer	O901 R1	0	0	0	0	0	0
	O901 R2	1	1	0	0	0	0
	O901 R3	1	1	0	0	0	0
	O901 Orig.	1	0	0	0	0	0

Inactivation of the hydrogen sulphide-producing enzymes by exposure

In the earlier experiments bacteria had been brought in contact with sulphur-containing substrates and antibiotics simultaneously. It was therefore not clear whether the antibiotics inactivated the H_2S-producing enzymes or whether the sulphur-containing substrates prevented the action of the antibiotics. To exclude any mutual influence the technique in the following experiments was changed. The bacteria were exposed for 24 hr. at 37° to different concentrations of antibiotics, washed twice in saline and then resuspended in the buffer + substrate solution. The experiments presented in Table 7 show that an exposure to 20,000 units penicillin/ml. inactivated the H_2S-producing enzymes of all strains under experiment. Aureomycin (1·0 mg./ml.) acted in the same way, but did not prevent hydrogen sulphide production from thiosulphate by *Brucella suis*. Chloramphenicol (1·0 mg./ml.) or streptomycin (0·1 mg./ml.) prevented H_2S-production by *B. melitensis* and two *Salmonella typhi* strains, while *Brucella abortus* and *B. suis* remained active.

Reactivation of H_2S*-producing enzymes by subcultivation*

The experiment presented in Table 8 was designed to show whether this inactivation was permanent or whether the H_2S-producing enzyme could be reactivated by subcultivation of the inactivated bacteria. The bacteria (two *Salmonella typhi* strains) were exposed to inactivating concentrations of antibiotics and then subcultured on plain agar, on casein hydrolysed by acid with or without 0·01 % cysteine, or with 0·05 % sodium thiosulphate. After one or two subcultures the H_2S-producing activity was again restored. Before the subcultures were started, all bacterial suspensions were counted by plating. Suspensions of *S. typhi* strains O901 and Ty2 contained almost the same number of viable organisms whether or not they had been kept in buffer solution or exposed to antibiotics; the respective counts for the two strains were $6{\cdot}5 \times 10^{11}$ and $8{\cdot}0 \times 10^{11}$ organisms/ml. This experiment proved, therefore, that although the antibiotics did not decrease the viability of resting cells, they damaged their H_2S-producing enzymes.

Table 7. *Inactivation of hydrogen sulphide producing enzymes by exposure of bacteria to antibiotics*

		Substrate									
		Cysteine					$Na_2S_2O_3$				
		Strain									
Antibiotic	Concentration (mg./ml.)	A	B	C	D	E	A	B	C	D	E
		Relative H_2S production									
Aureomycin	1·0	0	0	0	0	0	0	3	0	0	0
	0·1	0	3	3	0	3	0	3	3	0	2
Chloramphenicol	1·0	0	3	3	0	0	0	3	3	0	0
	0·1	0	3	3	0	2	0	3	3	0	0
Streptomycin	0·1	0	3	3	0	0	0	3	3	0	0
	0·01	3	3	3	3	3	3	3	3	0	2
	0·001	3	3	3	3	3	3	3	3	3	3
	(u./ml.)										
Penicillin	20,000	0	0	0	0	0	0	0	0	0	0
	2,000	2	0	0	3	3	0	3	3	0	0
	200	3	3	2	3	3	3	3	3	3	3

Bacteria $= 10^{11}$ ml. A = *B. melitensis*; B = *B. suis*; C = *B. abortus*; D = *S. typhi* (O901); E = *S. typhi* (Ty2).

Inactivation of the anti-desulphhydrase activity of penicillin by penicillinase

Penicillin (25,000 units/ml.) was left in contact for different periods with different concentrations of penicillinase (Nutritional Biochemicals Corporation, Cleveland, Ohio). Bacterial suspensions of 10^{11} organisms/ml. were exposed for 24 hr. to the products of this reaction. The organisms were removed from the penicillin + penicillinase mixture by centrifugation, washed in buffer solution and resuspended in cysteine + buffer solution. The results obtained with *Proteus vulgaris* and *Brucella abortus* (strain 19) are summarized in Table 9 which shows that a contact of 25,000 units penicillin/ml. with 0·2 % pencillinase for 2 hr. was long enough to inactivate the anti-desulphhydrase activity against *Proteus vulgaris*, while for *Brucella abortus* even 0·1 % penicillinase was sufficient. No further changes occurred when the activity of the penicillin and penicillinase mixtures was tested after 4 hr. contact.

Table 8. *Reactivation of hydrogen sulphide production by subculture on antibiotic-free medium*

Strain tested	Antibiotic (concentration/ml.)	Effect of 24 hr. exposure, H_2S production on		No. of subcultures required for reactivation on medium							
		Cysteine (C)	$Na_2S_2O_3$ (T)	I		II		III		IV	
				C	T	C	T	C	T	C	T
S. typhi O901	Penicillin 20,000 u.	0*	0*	1	1	1	2	1	1	1	1
	Streptomycin										
	0·2 mg.	0	0	1	2	2	2	2	2	2	1
	0·02 mg.	2	0	.	2	.	1	.	1	.	1
	Aureomycin										
	1·0 mg.	0	0	1	2	2	2	2	2	2	2
	0·1 mg.	0	0	1	2	1	2	1	2	2	2
	Chloramphenicol										
	1·0 mg.	0	0	1	2	1	2	1	1	1	1
	Control (Buffer)	3	3	.	.	.	.	.	.	.	.
S. typhi Ty2	Penicillin 20,000 u.	0	0	1	1	1	2	1	1	1	1
	Streptomycin										
	0·2 mg.	0	0	1	2	2	2	2	2	2	2
	Aureomycin										
	1·0 mg.	0	0	1	2	2	2	2	2	2	2
	0·1 mg.	0	0	1	2	1	2	1	2	1	1
	Chloromycetin										
	1·0 mg.	0	0	1	2	1	2	1	1	1	1
	Control (Buffer)	3	3	.	.	.	.	.	.	.	.

Medium I = plain agar; medium II = acid hydrolysed casein; medium III = as II with cysteine 0·01 %; medium IV = as II with $Na_2S_2O_3$ 0·05 %.

* Figures in these columns have the same significance as in Table 1.

C = cysteine as substrate; T = thiosulphate as substrate.

Table 9. *Inactivation of anti-desulphhydrase activity of penicillin by penicillinase*

Test performed with *B. abortus* and *P. vulgaris* on cysteine (10^{11} cells/ml.).

Time of reaction (hr.)	Test strain	Concentration of penicillinase				
		0·5 %	0·2 %	0·1 %	0·05 %	0 (control)
		Relative H_2S production after contact with the product of the penicillin-penicillinase reaction				
2	*P. vulgaris*	2	2	0	0	0
	B. abortus	2	2	2	0	0
4	*P. vulgaris*	2	2	0	0	0
	B. abortus	2	2	2	0	0

The minimal time of contact with penicillin required for the inactivation of bacterial desulphhydrases

Organisms (10^{11}/ml.) were exposed for different times to 25,000 units penicillin/ml. at 37°, centrifuged, washed in buffer solution, resuspended in substrate + buffer solution and examined for H_2S production. The results, summarized in Table 10, show that time intervals from 5 to 20 hr. had to elapse before complete inactivation was reached. After an exposure to penicillin for 20 hr. all five organisms were completely inactivated.

Table 10. *Inactivation of* H_2S *formation by bacterial suspensions exposed to penicillin*

Suspensions: 10^{11} cells/ml. Penicillin 25,000 u./ml. Temp. 37°.

Substrate tested	Exposure (hr.)	Relative H_2S production observed with strain				
		A	B	C	D	E
Cysteine	2·5	2	3	3	3	3
	5·0	1	3	3	3	1
	10·0	0	3	3	3	0
	15·0	0	2	2	0	0
	20·0	0	0	0	0	0
	Control not exposed	3	3	3	3	3
Sodium thiosulphate	2·5	0	2	2	3	3
	5·0	0	2	0	3	3
	10·0	0	0	0	3	3
	15·0	0	0	0	0	0
	20·0	0	0	0	0	0
	Control not exposed	1	3	2	3	3

A = *S. typhi*; B = *P. vulgaris*; C = *P. morganii*; D = *B. abortus* (strain 19); E = *B. abortus* (strain 2308).

Reactivation of the desulphhydrase activity by penicillinase

The experiments described above showed that under the conditions used, the anti-desulphhydrase action of penicillin was destroyed by penicillinase within 2–4 hr. An attempt was made to determine the minimal time of contact between penicillin and bacteria required to inactivate the desulphhydrase, by addition of penicillinase at different times after mixing penicillin and bacteria. The addition of the penicillinase effected a reactivation of the bacterial desulphhydrases while washing left the bacteria inactive.

Table 11 shows that the bacterial desulphhydrase activity was not reactivated by addition of the substrate to the penicillin + bacteria mixture or by washing and resuspension in the substrate + buffer solution. However, when substrate + penicillinase were added, some traces of hydrogen sulphide

Table 11. *Penicillinase reactivation of penicillin-inactivated suspensions*

Suspension: 10^{11} bacteria/ml. exposed 20 hr. to 25,000 u. penicillin/ml.

Treatment of the bacteria after 24 hr. exposure to penicillin	Relative H_2S production of		
	S. typhi	*P. vulgaris*	*B. abortus*
(1) Cysteine and penicillinase (0·5 %) added	1	1	1
(2) Cysteine added	0	0	0
(3) Bacteria washed, then cysteine and penicillinase (0·5 %) added	2	2	2
(4) Bacteria washed, then cysteine added	0	0	0

appeared after 48 hr. When the bacteria were washed and the substrate and penicillinase added, the reactivation was complete and within 24 hr. hydrogen sulphide was produced. It seems, therefore, that centrifugation and washing alone did not remove all traces of penicillin. On the other hand, washing followed by penicillinase treatment reactivated the bacterial cells, proving that the changes exerted by penicillin were reversible. Washing alone, even repeated five times, did not exert this effect.

Since the penicillinase solutions were partly inactivated by filtration through Seitz filters, we compared in the following experiments the reactivating action of different concentrations of both filtered and non-filtered penicillinase solutions. Table 12 shows that with 0·2 and 0·1 % non-filtered, and with 0·5 and 0·2 % filtered solutions of penicillinase no reactivation of cysteine desulphhydrase was obtained unless the bacteria were washed and then brought in contact with penicillinase + cysteine. Similar results were obtained with sodium thiosulphate instead of cysteine. Higher concentrations of non-filtered penicillinase solutions were not employed, since higher concentrations of this enzyme preparation alone when brought in contact with cysteine themselves produced hydrogen sulphide after 48 hr. In order to exclude this source of error the maximal concentration used was 0·2 %.

Table 12. *Reactivating effect of different types of penicillinase solutions*

Suspensions 10^{11} bacteria/ml. inactivated by 25,000 u. penicillin/ml.

	Penicillinase		Relative H_2S production by strain				
Treatment after inactivation	Type	Concentration (%)	A	B	C	D	E
Penicillinase added	Not filtered	0·2	0	0	1	0	0
		0·1	0	0	0	0	0
	Filtered	0·5	0	0	0	0	0
		0·2	0	0	0	0	0
Bacteria washed and penicillinase added	Not filtered	0·2	2	2	2	2	2
		0·1	1	2	2	2	1
	Filtered	0·5	0	2	2	2	0
		0·2	0	2	1	2	0

A = *S. typhi*; B = *P. vulgaris*; C = *P. morganii*; D = *E. coli*; E = *B. abortus* (strain 19).

Reactivation of the desulphhydrase activity by bacterial extracts

Two bacterial extracts, using McIlwain's (1948) method, were prepared from *Proteus vulgaris* and *Brucella abortus* (strain 19). The respective protein content of the extracts was 6·8 and 5·6 mg./ml. When varying quantities of each extract were brought in contact with cysteine or sodium thiosulphate, hydrogen sulphide was produced as follows:

Extract concentration (%) ...	50	20	10	5	2
Proteus extract					
Cysteine	3	1	1	1	0
Sodium thiosulphate	3	1	0	0	0
Brucella extract					
Cysteine	1	1	1	1	0
Sodium thiosulphate	0	0	0	0	0

In order to avoid error from this source of hydrogen sulphide production, the extracts were added to the inactivated and washed bacteria in 1 % concentrations on cysteine, and 10–20 % on sodium thiosulphate.

As in the earlier experiments bacterial suspensions containing 10^{11} organisms/ml. were left in contact at 37° with 25,000 units penicillin/ml. for 24 hr. The bacteria were then centrifuged, washed and resuspended in the substrate + buffer solutions, and bacterial extracts were added.

The low concentrations of extract only caused a slight reactivation of the hydrogen sulphide production by penicillin-inactivated bacteria (Table 13). This desulphhydrase activity was eliminated by heating extracts at 50° for 30 min. while their reactivating power on inactivated bacteria was still intact. 10 μg./ml. of pyridoxal phosphate also exerted a slight reactivating activity.

Table 13. *The reactivation of penicillin-inactivated suspensions*

Suspensions 10^{11}/ml. inactivated by 25,000 u./ml.

Extracts from	Concentration (%)	Substrate tested	Relative H_2S production by strains A	B	C	D
B. abortus	1·0	Cy.	0	0	0	1
	20·0	T.	0	0	1	3
P. vulgaris	1·0	Cy.	0	0	1	1
	10·0	T.	1	0	1	1
	Controls without extract	Cy.	0	0	0	0
		T.	0	0	0	0

A = *P. vulgaris*; B = *P. morganii*; C = *E. coli*; D = *B. abortus* (strain 19). Cy. = cysteine; T. = Sodium thiosulphate.

These two activating factors, extracts heated at 50° for 30 min. and pyridoxal phosphate, were employed in the following experiments. Since the desulphhydrase activity of the extract was inactivated, 20 % concentrations were used for the reactivation of the bacteria. The bacterial inocula and the concentration of penicillin employed for inactivation were the same as in the experiment shown in Table 13. Table 14 summarizes an experiment of this type. Pyridoxal phosphate exerted only a slight reactivating effect on the bacteria tested on cysteine. In comparison with this weak action, the reactivation by the bacterial extract was much stronger and in the presence of both extract and pyridoxal phosphate, hydrogen sulphide production was accelerated.

The next experiment was carried out with *Proteus vulgaris*. Extract was prepared from *Brucella abortus* and was heated at 50° for 1 hr. The reactivation was tested on cysteine and different inocula and different concentrations of the two reactivating factors were employed. The inactivation of the bacteria was carried out as in the earlier experiments.

A control showed that the extract and pyridoxal phosphate either separately or together did not produce hydrogen sulphide on cysteine + buffer solution without the inactivated bacteria. The experiment summarized in Table 15 showed that inocula of 10^{11} and 10^{10} organisms/ml. were reactivated by

2·5–20 μg./ml. of pyridoxal phosphate, while this effect was not produced with an inoculum of 10^9 organisms/ml.

Table 14. *The reactivating effect of heated proteus extract and of pyridoxal phosphate*

Suspensions 10^{11}/ml. inactivated by 25,000 u. penicillin/ml.

Quantities added			Relative H_2S production by strain			
Heated *P. vulgaris* extract (%)	Pyridoxal phosphate (μg./ml.)	Substrate tested	A	B	C	D
20	0	Cy.	3	3	3	2
20	10	Cy.	3	3	3	3
20	0	T.	2	2	2	0
20	10	T.	3	3	2	0
0	0	Cy.	0	0	0	0
0	10	Cy.	1	1	0	0
0	0	T.	0	0	0	0
0	10	T.	0	0	0	0

Strains A–D: the same as in Table 13. Cy. = cysteine; T. = sodium thiosulphate.

Table 15. *Reactivation of inactivated* P. vulgaris *suspensions by pyridoxal phosphate and* B. abortus *extract*

	Proteus vulgaris suspension (cells/ml.)					
	10^{11}		10^{10}		10^9	
Concentrations of the brucella extract (%)	P.	NP.	P.	NP.	P.	NP.
	Relative H_2S production observed					
20	3	2	3	2	3	3
10	3	2	2	2	3	2
5	2	1	2	1	2	1
2	2	1	2	1	2	1
1	2	0	2	0	2	1
Control without extract	2	0	2	0	0	0

P. = 2·5–20 μg./ml. pyridoxal phosphate added; NP. = pyridoxal phosphate not added.

In higher concentrations of the bacterial extract and in the absence of pyridoxal phosphate, all inocula produced hydrogen sulphide within 24 hr., while the lower concentrations were relatively inactive and did not exert much effect. It seems, therefore, that for a complete reactivation of small inocula of *Proteus vulgaris* (10^9 organisms/ml.) two different methods may be employed: high concentrations of bacterial extracts or low concentrations of bacterial extracts simultaneously with pyridoxal phosphate. However, the reactivation of suspensions containing 10^{10}–10^{11} organisms/ml. can also be effected by pyridoxal phosphate alone.

Suspensions containing 10^{11} organisms/ml. were kept in the ice-box for 14 days to autolyse, then heated at 100° for 1 hr., and centrifuged until the supernatant fluid became completely clear. 50 % concentrations of these extracts exerted some reactivating effects. An extract prepared from *Brucella abortus* reactivated *Proteus morganii* and *Escherichia coli* on cysteine and sodium thiosulphate, but failed to reactivate *Salmonella typhi* and *Brucella abortus*. While these reactivating effects were observed, the extracted bacterial suspensions failed to exert such an effect.

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The Filamentous Forms of *Bacillus megaterium*

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SUMMARY: The fine filaments of *Bacillus megaterium* are shown to arise from large basal cells: they have paired nuclear bodies within walls differing in composition from those of the normal vegetative cells. No evidence was found for any sexual fusion of these filaments.

Young cultures of *Bacillus megaterium* often contain filamentous forms, especially when grown on blood-containing media. These differ from the vegetative cells typical of the growth cycle described by the author (Bergersen, 1953). DeLamater (1951), and DeLamater & Hunter (1953), described fusion tubes by means of which a diploid phase was instituted in *B. megaterium*. These tubes were fine filaments which fused with large haploid cells and were supposed to transfer nuclear material from one cell to another. Bisset (1953) criticized this work and stated that the filaments involved were septate, and it was therefore difficult to understand how they could function as fusion tubes. The present work was undertaken as a study of the fine filaments, with particular reference to their cytology and behaviour in living cultures.

MATERIALS AND METHODS

The bacteria used were strains of *Bacillus megaterium* isolated in Dunedin and also NCTC strain 7581. These strains were maintained on nutrient agar slopes and subcultured in meat extract broth at 35° overnight before being used. In all the experiments the inocula were taken from 12 to 18 hr. broth cultures. When human serum (4 %, v/v) was added to a nutrient agar base, copious fine filaments were produced in 3–5 hr. at 35°. Filament production was also examined on serum-free media using inocula from serum-free broth and from human blood agar.

Slide cultures were prepared by pouring the melted agar media thinly in Petri dishes: when it had set, the inocula were spread evenly on the surface, care being taken that heavy loop tracks were not left: strips 1·5 × 0·5 cm. were cut from the agar and mounted on sterile slides under thin coverslips 1·5 cm. square. The preparations were then partially sealed with sterile vaseline: this minimized drying effects, while at the same time an air gap was left on either side of the agar strip to maintain aeration during the time of observation. These slide cultures were observed continuously by means of a phase-contrast microscope mounted in a specially built incubator kept at 35°.

Cytological preparations were made on coverslips from impression films from agar cultures. These were fixed in osmic acid vapour and stained by the HCl-Giemsa method for nuclear structure. Cell walls were stained by Victoria blue 4R after treatment with 10 % tannic acid for 10–15 min. The method of Post & Laudermilk (1942) was adapted as follows for studies of the cell walls of the filaments. Fixed impression films were covered with a solution of iodine (2 %) in potassium iodide (5 %) for 10 sec., blotted dry and mounted in saturated aqueous lithium chloride solution.

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OBSERVATIONS

Slide cultures. In these cultures the origin and growth of the fine filaments were carefully followed in a large number of developing microcolonies. When the inocula were taken from 12 to 18 hr. broth cultures, the sequence of developments was as follows. During the first hour the usual lag-phase enlargement of the bacteria proceeded and the first one or two divisions occurred. The cells which produced the filaments then enlarged slightly to form a basal cell and a tapering extension was produced at one end. The basal cell became divided from the developing filament, which after 2–3 hr. was sometimes 3 times the length of the parent cell, but more often was long and tenuous, often becoming lost among other bacilli of the growing colony. At first the filaments showed no sign of division, but after 3 hr. some fragmentation occurred at the basal end. Often the parent cell divided several times before producing the filament, and on several occasions one of the resulting cells divided with two septa very close together and a very small cell resulted (Pl. 1, figs. 1, 2). Some of the filaments suddenly lost contrast after 2–3 hr. and remained as 'ghosts' with very little structure. Most of them, however, retained their contrast until they became overgrown and lost, but the filaments were always paler than the actively dividing vegetative cells. As they extended, the fine filaments grew between the cells of the growing microcolonies often twisting and coiling and otherwise showing considerable flexibility. The tips of many of them came to rest against the walls of large cells and seemed to stick to them. This was at first thought to be a fusion of the filament and the large cell. After further examination this seemed doubtful since an apparently fused filament often would break away and stick to a cell on the other side (Pl. 1, figs. 1, 2). On one or two occasions the appearance of a fusion was more convincing but there was difficulty in distinguishing fusion from mere contact, in phase-contrast studies.

Both slide cultures and cultures grown on agar plates showed that a basal nutrient agar medium plus human serum (4 %, v/v) gave many fine filaments during the first 4 hr. of incubation. A few were seen on the basal medium without serum, but these were small and often lysed. When the inoculum was taken from blood agar cultures and then grown on the basal medium more filaments were produced. It seems clear that these forms are therefore produced as the result of a nutritional stimulus as previously reported by DeLamater (1951) for fusion tubes. Slight differences in the extent to which filaments were formed were observed in the various strains studied, but growth and behaviour seemed uniform.

Nuclear stains. These preparations showed that the filaments contained pairs of nuclear bodies throughout their length. Sometimes a septum divided the terminal part. The basal cells had a complex arrangement of nuclear material, stained blue by Giemsa, and also a number of pink granules resembling the metabolically active granules described as growing points (Bergersen, 1953) in vegetative cells. These granules confuse the black and white photomicrographs in Pl. 1, but the generalized lay-out of nuclear material is shown in the diagram (Fig. 1). This complex nuclear apparatus was most noticeable in basal cells whose filaments were still attached or divided by only one septum. Older filaments became completely separated from the basal cells which often lysed. The thick proximal end of the filaments contained a bar of nuclear material composed of a zigzag of poorly defined bars from which the paired bodies seemed to be derived.

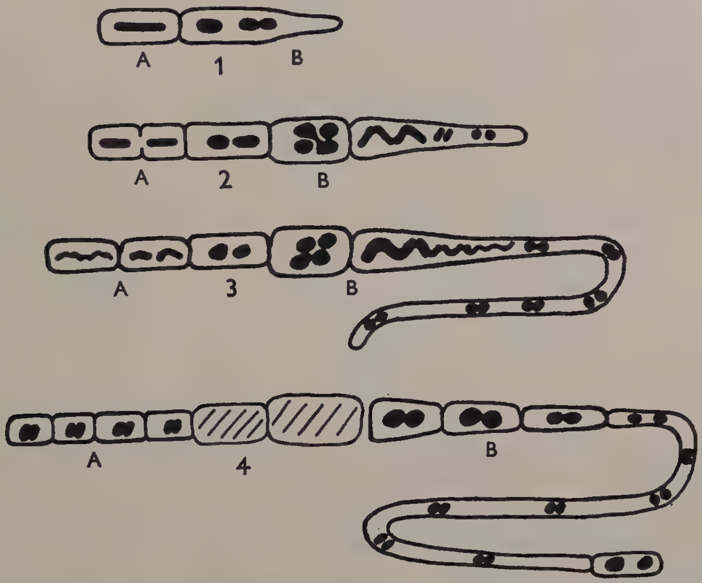

Fig. 1. A diagrammatic representation of the formation of fine filaments, B, and the corresponding stages of vegetative growth A. 1, the first stage; 2, the basal cell divided from the filament; 3, zigzag nuclear material producing pairs of nuclear bodies in the filament; 4, septation of the filament begins.

Cell-wall stains. Victoria blue 4R stained preparations showed the filaments to be mainly non-septate, although the basal cells and beginning of the filament were divided in older cultures (Pl. 2, figs. 9–12). With Post & Laudermilk's stain photographs of organisms were unsuitable for reproduction, but direct visual examination was satisfactory. The cell walls of large vegetative organisms stained a brownish yellow, typical of certain types of cellulose, but the walls of the fine filaments remained unstained. This suggests a different chemical structure in the walls of these forms.

DISCUSSION

From these studies it seems that the fine filaments of *Bacillus megaterium* are cell types produced in response to a nutritional stimulus provided by a factor in human serum; this is in agreement with DeLamater (1953). They are produced from enlarged basal cells whose nuclear material seems to undergo considerable rearrangement. The filaments themselves have paired nuclear bodies, whereas previous work has shown that vegetative filaments have a fusion nucleus which breaks up to form the nuclei of separate cells; these may continue in the vegetative cycle (Bergersen, 1953) or in some groups proceed to spore formation (Kleinberger-Nobel, 1945). The lack of cellulose-like material in the cell wall may explain the flexibility of the fine filaments, which is often considerable. It is the opinion of the present author that these fine filaments are those described by DeLamater & Hunter (1953) as fusion tubes. Evidence was sought during the present work to support the hypothesis of DeLamater & Hunter that these forms may be concerned in a sexual phase in *B. megaterium*. In slide culture apparent fusion between filaments and large cells was seen, without however any evidence from the cytological preparations for any dissolution of the cell walls between apparently fusing elements (Pl. 2, figs. 10, 11). It cannot be said at this stage that true fusion did not occur; but it was not observed during the present work although slide cultures were continuously observed for long periods and although large numbers of cytological preparations were made and each one exhaustively studied. Some bacilli were seen with short filaments resembling DeLamater's photographs of 'fusion tubes'. From the present work it appears clear that these were the beginnings of filament formation and not the result of fusion (Pl. 1, figs. 5–8). The true function of the fine filaments in cultures on agar media is obscure but no evidence was found for a sexual fusion between filament and large bacillus.

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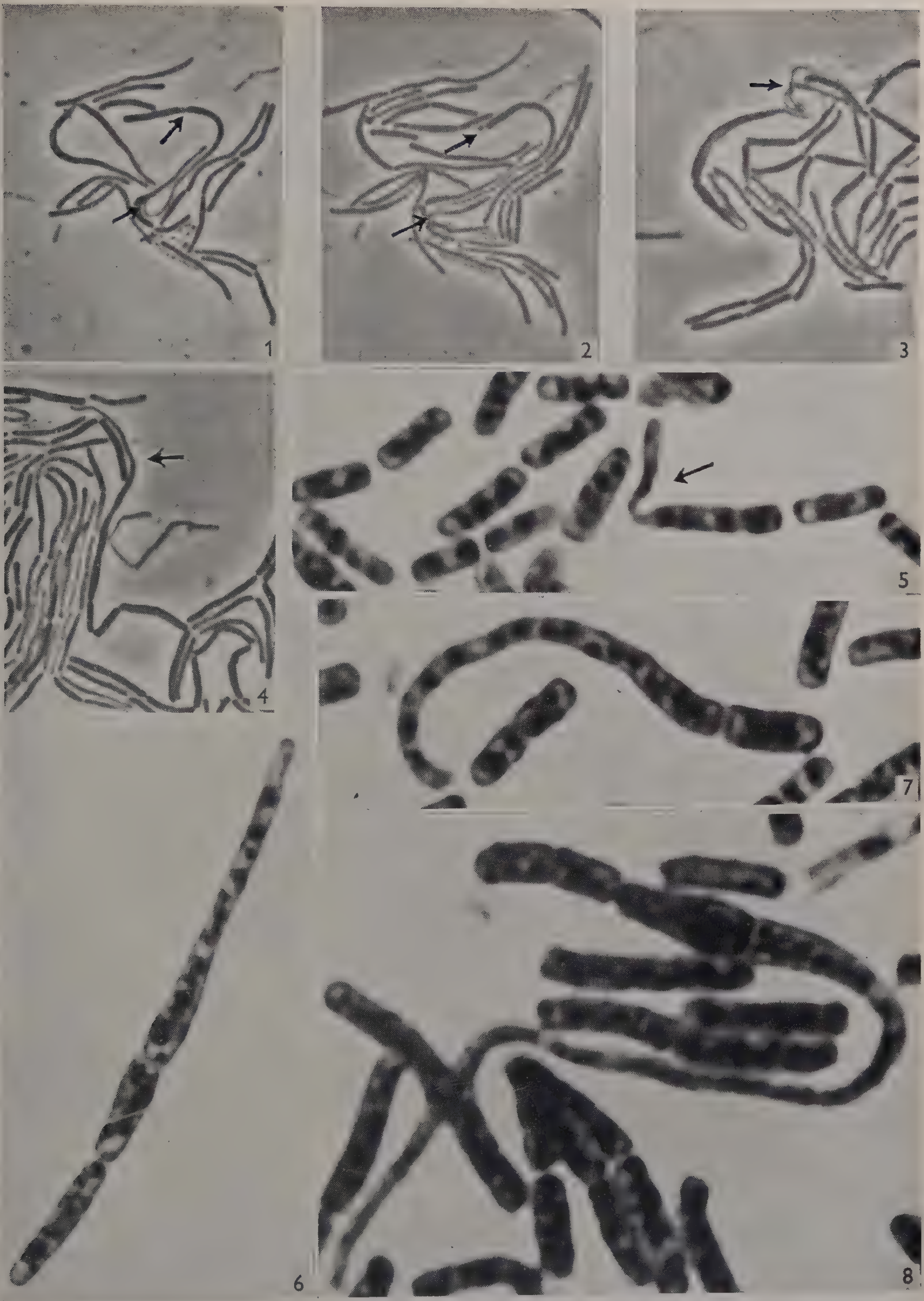
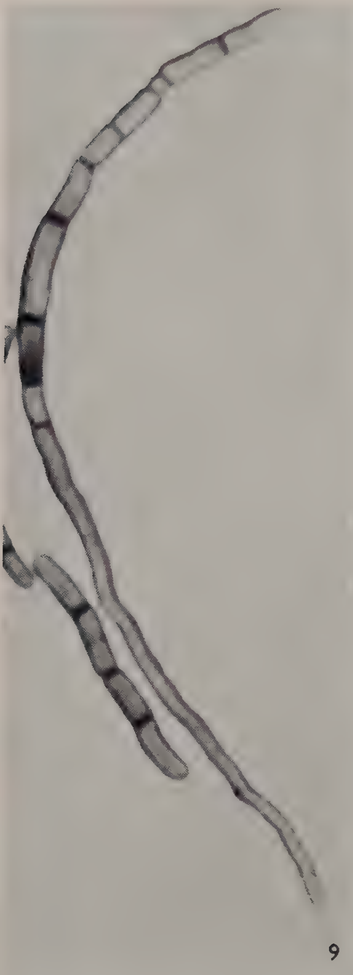


F. J. Bergersen—Filamentous forms of *Bacillus megaterium*. Plate 1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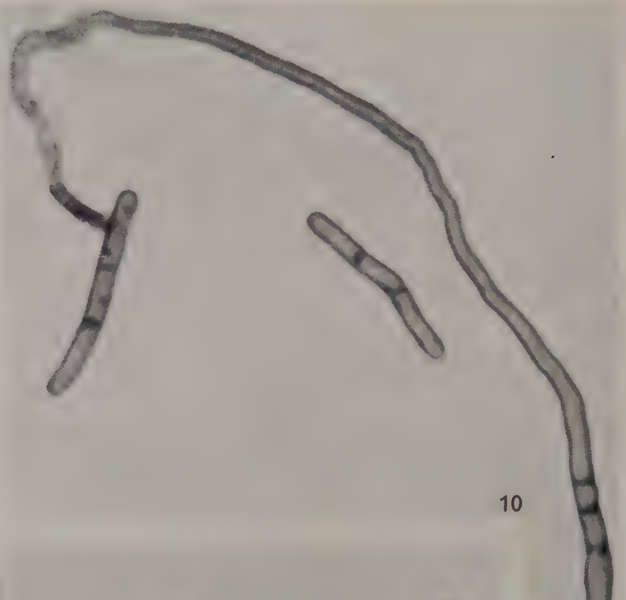
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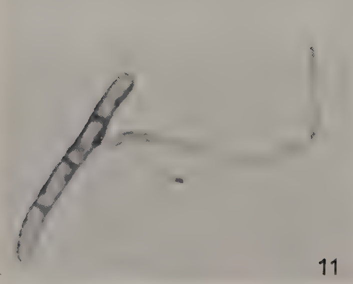
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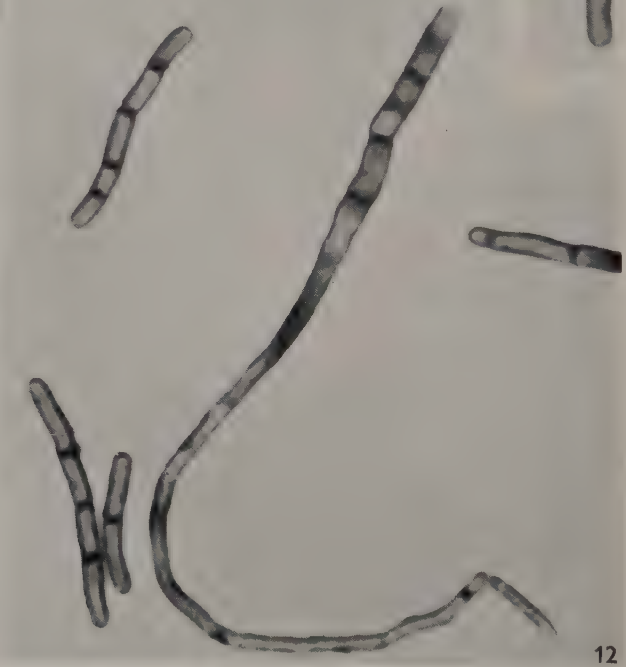
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F. J. Bergersen—Filamentous forms of *Bacillus megaterium*. Plate 2

EXPLANATION OF PLATES

Plate 1

Figs. 1, 2. Two photographs of the same field taken 15 min. apart. The upper arrow shows the formation of a very small cell and the lower arrow shows an apparently fusing filament which becomes stuck to another cell on the opposite side. Phase contrast. ×450.

Fig. 3. A much coiled, flexible filament. Phase contrast. ×450.

Fig. 4. A typical fine filament and its large basal cells.

Fig. 5. The beginning of filament formation. A stage resembling 'fusion tubes'. HCl-Giemsa. ×3500.

Figs. 6–8. Later stages than fig. 5: swelling basal cells and paired nuclear bodies in the filament HCl-Giemsa. ×3500.

Plate 2

Victoria Blue 4R stained impression smears. ×2000.

Fig. 9. Non-septate filament, basal cell and vegetative cells.

Figs. 10, 11. Apparently fusing filament and large cell. No dissolution of cell wall.

Fig. 12. Filament with septate tip and basal cells.

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The Formation of Diazotizable Amine and Hypoxanthine by a Yeast: Possible Implications in the Biosynthesis of Purines

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SUMMARY: A diazotizable amine and hypoxanthine accumulate in cultures of Yeast 47 grown in a defined medium containing added methionine and suboptimal biotin. Amine accumulation is depressed by the presence either of added adenine, or of certain amino acids, of which aspartate, norleucine, norvaline and threonine are the most effective. The amine differs from 4-amino-5-imidazolecarboxamide and the corresponding amidine. Spectrophotometric measurements suggest that the amine lacks the completed 6-membered ring structure of the purine skeleton, but it has biological activity in lieu of adenine for the adenine-requiring Yeast 19, and of guanine for a strain of *Lactobacillus brevis*.

Hypoxanthine substitutes for adenine in the growth of Yeasts 19 and 47, and for adenine + guanine in *Lactobacillus brevis*, which suggests that hypoxanthine is a bioprecursor of both adenine and guanine. The accumulation of hypoxanthine in cultures of Yeast 47 may thus be the result of a synthetic block, conditioned by biotin deficiency, which prevents the conversion of hypoxanthine to adenine and guanine. The possible relationships between diazotizable amine and hypoxanthine are discussed.

Chamberlain, Cutts & Rainbow (1952) reported the conditions under which Yeast 47 formed pigment and accumulated a diazotizable amine in liquid medium. This paper reports a study of the constituents present in amine-containing concentrates prepared from culture filtrates of Yeast 47.

METHODS

Test organisms. Three organisms were used:

Saccharomyces cerevisiae (Yeast 47). For growth in a defined medium, Yeast 47 required added biotin and *p*-aminobenzoic acid (*p*-AB). The *p*-AB was replaceable, at least in part, by relatively large concentrations of adenine, methionine and histidine (Cutts & Rainbow, 1950).

Schizosaccharomyces octosporus (Yeast 19). For growth in a defined medium, Yeast 19 required added adenine, methionine and histidine, but not *p*-AB (Northam & Norris, 1951).

Lactobacillus brevis. This was the heterofermentative lactobacillus used by Chamberlain *et al.* (1952). For growth it required added adenine, guanine and pteroylglutamic acid (PGA).

Cultures of the yeasts were maintained as described by Cutts & Rainbow (1950). *Lactobacillus brevis* was maintained as stabs in 10 % malt wort agar supplemented with 0·1 % Difco yeast extract and 0·5 % L-arabinose.

Media. Basal medium A (for Yeast 47) was that described by Cutts & Rainbow (1950), but biotin was omitted, 0·10 μg. *p*-AB/ml. was added and the pH adjusted to 4·8.

Basal medium B was as A, but *p*-AB was omitted and the following additions (μg./ml.) made: D-biotin (0·1), adenine (50), DL-methionine (100), L-histidine HCl (100). This medium was used for tests involving Yeast 19 and for Yeast 47 in *p*-AB-free medium.

Basal medium C (for *Lactobacillus brevis*) was as follows. Per 100 ml. final volume, in g.: glucose, 2·0; $CH_3COONa.3H_2O$ (A.R.) 3·2; casein acid hydrolysate ('vitamin free', Allen and Hanbury) 1·0; $(NH_4)_2SO_4$ (A.R.) 0·3; NaCl (A.R.) 0·5; in mg.: L-cystine, 20; DL-tryptophan, 20; KH_2PO_4 (A.R.) 50; K_2HPO_4 (A.R.) 50; $MgSO_4.7H_2O$ (A.R.) 10; $MnSO_4.4H_2O$ (A.R.) 0·5; $FeCl_3$ (A.R.) 0·1; in μg.: thiamine (100); pyridoxin (100); nicotinic acid (100); calcium D-pantothenate (100); riboflavin (50); *p*-AB (20); PGA (10); D-biotin (0·02). Adjustment was made to pH 6·5, but, after sterilizing by heating in the autoclave momentarily to 10 lb. pressure, the pH fell to 6·2, the optimum for *L. brevis* in this medium.

Inocula were prepared as follows:

Yeast 47: grown up in basal medium A containing 0·1 mμg. D-biotin/ml., other details being according to Cutts & Rainbow (1950).

Yeast 19: as for Yeast 47, but grown up in basal medium B.

Lactobacillus brevis: grown up at 28° for 16–24 hr. in basal medium C fortified with 10 μg. of adenine and of guanine/ml. Harvesting and washing the cells was as above. One drop (*c.* 20 μl.) of the final suspension of the washed cells was used/3 ml. of medium to inoculate auxanograms. For tube tests, three drops of the final suspension were shaken with 6 ml. sterile 0·85 % saline and one drop of this dilute suspension used to inoculate each tube.

Tube tests (6 ml. cultures) were carried out as described by Northam & Norris (1951). Incubation was at 25° (yeasts) or 28° (*Lactobacillus brevis*).

Auxanograms were carried out in the appropriate basal medium at 28°, according to Cutts & Rainbow (1950), except that, in most cases, substances for test were applied to the agar as circular paper disks cut from papergrams with a sharp cork borer, after the position of the spot chosen for test had been located as indicated below.

Diazotizable amine was determined as described by Chamberlain *et al.* (1952), but omitting the acetylation procedure.

Chromatography. Chromatograms were run on Whatman no. 4 special chromatography paper (22½ in. long) in a glass cabinet maintained at 23–25°, the solvent being *n*-butanol + acetic acid + water (Woiwod, 1949). Test materials were applied as 10 μl. spots, larger spots (up to 50 μl.) being obtained, if necessary, by successive applications of 10 μl. portions, with drying between applications. Under these conditions, the run covered the length of the paper in 12½ hr., but no ill effects resulted when the papers were left for 18 hr. After runs, papers were dried in a current of warm air and compounds then detected as follows: purines by ultraviolet (UV) photography (Markham & Smith, 1949); amino acids by the ninhydrin reaction (Woiwod, 1949); diazotizable amine by the Bratton & Marshall (1939) reaction. For the latter reaction the reagents were made up in ethanolic solution to ensure rapid drying after the application of each spray to minimize spreading of the spots. The first spray consisted of a mixture of 1 vol. aqueous 4N-HCl + 1 vol. 0·2 % sodium nitrite in 80 % (v/v) ethanol. After 3 min., 2 % ammonium sulphamate in 75 % (v/v) ethanol was sprayed on, followed rapidly by 0·1 % N-(1-naphthyl)-ethylenediamine 2HCl in absolute ethanol. Diazotizable amine showed as a pink spot after this treatment.

To examine concentrates of diazotizable amine, three identical spots were chromatographed side by side at one edge of the paper, suitable reference spots (e.g. authentic purines) being included with each run. After the run the complete papergram was examined for purines and two strips, corresponding to the outer spots of concentrate, cut from the paper for further examination, one for amino acids and the other for diazotizable amine. In this way, the positions of amino acids, UV-absorbing substances and diazotizable amine were established on the main papergram, which, being untreated by reagents, was utilizable for biological tests by auxanography.

Spectrophotometry. Measurements were made in 1 cm. quartz cells with the Unicam SP 500 quartz spectrophotometer. Distilled water was used as reference liquid, except when papergram eluates in 0·1 N-HCl were measured. In such cases, a blank eluate in 0·1 N-HCl, obtained from an unoccupied area of the papergram, was used.

'Aldehyde-free' ethanol was prepared by refluxing absolute ethanol for 1 hr. in the presence of 2:4-dinitrophenylhydrazine (1 g./100 ml.) and a trace of H_2SO_4. The fraction which distilled at 77·5–78·5° was collected for immediate use.

EXPERIMENTAL

Preparation of amine concentrates

To produce diazotizable amine in quantities sufficient for experimental work, the following procedure was adopted. Two portions (each 1 l.) of medium A fortified with 0·02 mμg. D-biotin and 500 μg. DL-methionine/ml., were sterilized (15 lb./sq. in. momentarily) in 6 l. Erlenmeyer flasks loosely plugged with cotton-wool. Each flask was inoculated with 6 ml. of inoculum of Yeast 47 and incubated at 21° for 6 days. The pink cell crop was filtered off (Green's paper, no. 503), the filtrates (pH 4·4) combined, and a freshly prepared solution of 8 g. mercuric acetate in 150 ml. water added slowly with stirring. The precipitate was allowed to settle overnight at 4° and collected by decantation followed by centrifugation. After washing twice with water, the precipitate was dispersed in 130 ml. 0·1 N-H_2SO_4 and decomposed by a current of H_2S passed for 1 hr. Excess H_2S was removed by a current of air, a previously determined volume of barium hydroxide solution added to neutralize the H_2SO_4 and the mixed precipitates of HgS and $BaSO_4$ centrifuged off. The supernatant was evaporated *in vacuo* below 40° to 10 ml. and treated with 90 ml. of 'aldehyde-free' ethanol. After standing for 2 hr. at 4°, the precipitate was centrifuged off, ethanol removed from the supernatant by evaporation *in vacuo* to small volume and the final solution made up to 25 ml. This solution (pH 4·0), containing about 50 % of the amine present in the original culture filtrate, was used in the subsequent experimental work. The most significant losses of amine in this preparation appeared to occur during the decomposition of the mercury precipitate.

Chromatography of amine concentrates

Table 1 shows the R_F values of diazotizable amine, purine and amino acids detected in amine concentrates by paper chromatography, together with the R_F values of authentic reference compounds. The predominant constituents of the amine concentrate thus revealed were:

Diazotizable amine: this spot was positionally distinct from the amino acids and purines present, with the possible exception of guanine. A faint UV-absorbing spot was visible in the amine position, which may have represented the amine itself, or very small amounts of guanine. The first possibility was regarded as the more likely from the spectrophotometric evidence (see below). No other diazotizable material was visible on the papergrams.

The UV-absorbing spot was positionally distinct from amine and corresponded closely with hypoxanthine. Its size and intensity indicated the presence of about 20 μg. hypoxanthine/30 μl. amine concentrate. There was some overlap between this spot and the large ninhydrin-positive spot ((*c*) below). The latter, however, was not UV-absorbing.

The large ninhydrin-positive spot corresponded in position to methionine. Methionine is precipitated by mercuric salts and would therefore be concentrated along with diazotizable amine owing to its presence in the original medium.

Table 1. *R_F values of substances present in concentrates*

Identical conditions for both chromatograms; temperature, 23–25°; duration of run, 12 hr.; solvent, *n*-butanol + acetic acid + water; spots: amine concentrate, 30 μl., reference substances, 20 μg.

	Description of spot	Chromatogram		Intensity of spot
		(1)	(2)	
Amine concentrate	Ninhydrin positive	0·11	0·13	Weak
		0·15	0·17	Weak
		0·20	0·22	Weak
		0·23	0·25	Weak
		0·33	0·34	Weak
		0·50	0·52	Very strong
	Diazotizable	0·36	0·38	Strong
	UV-absorbing	0·49	0·50	Very strong
Reference substances	Guanine	0·39	0·39	Strong
	Xanthine	0·42	0·42	Strong
	Hypoxanthine	0·49	0·50	Strong
	DL-Methionine	0·50	0·52	Strong
	Adenine	0·61	0·62	Strong

Neither amine nor the UV-absorbing constituent (UVAC) was detectable in concentrates prepared similarly from cultures of Yeast 47 which contained optimal biotin (1 mμg./ml.) added to the medium. With suboptimal biotin (0·02 mμg./ml.) but no added methionine, both amine and UVAC were formed, only in much smaller amounts than when methionine was added. When methionine was not added the only source of methionine was that synthesized by the cells themselves.

Spectrophotometry of amine concentrates

Absorption curves were measured over the range 200–300 mμ. on solutions of (i) pure reference substances (10 μg./ml.); (ii) as (i), but obtained by cutting the appropriate spots from papergrams and eluting with 0·1 N-HCl; (iii) the chief spots from amine concentrates, isolated from papergrams as in (ii).

The results (Table 2) confirmed the identity of UVAC with hypoxanthine and indicated that the diazotizable amine was not contaminated with detectable amounts of guanine. The reference purines and pyrimidines gave characteristic end-absorption at *c.* 200 mμ., with peak absorption at 248–284 mμ. By contrast, diazotizable amine, methionine and histidine showed end-absorption but no peak within the latter range.

Table 2. *Values of* $\lambda_{\max.}$ *for reference substances and constituents of amine concentrate*

Stock solutions of reference substances, 10 μg./ml. in 0·1 N-HCl, except DL-methionine, 50 μg./ml.; papergram extracts, 5 ml. of eluates in 0·1 N-HCl from 5 × 30 μl. spots of amine concentrate.

	$\lambda_{\max.}$(mμ.) Stock solutions		Papergram extracts	
	(1)	(2)	(1)	(2)
Amine concentrate:				
UV-absorbing spot	—	—	<200	248·0
Diazotizable amine	—	—	206	No peak
Reference substances:				
Adenine	204	262·5	205·5	262·5
Guanine	202·5	248·5	204	248·5
Hypoxanthine	<200	248·0	<200	248·0
Uric acid	230	283·5	—	—
Xanthine	202	265·0	—	—
Cytosine	209·5	275·5	—	—
Thymine	206	265·0	—	—
Uracil	204	258·5	—	—
DL-Methionine	204	No peak	—	—
L-Histidine HCl	210	No peak	—	—

Biological activity of constituents of amine concentrates

Auxanograms. Circles corresponding to constituents of the amine concentrate and to known reference substances (10 μg.) were cut from papergrams and tested for biological activity as substitutes for adenine in the growth of Yeasts 19 and 47 and for adenine and guanine in the growth of *Lactobacillus brevis*. For *L. brevis*, three auxanograms were used, involving medium C supplemented (*a*) with 10 μg. guanine/ml. (to test capacity to substitute for adenine), (*b*) with 10 μg. adenine/ml. (to test capacity to substitute for guanine), or (*c*) with no addition (to test capacity to substitute for adenine + guanine).

The following substances were tested on each plate: adenine, hypoxanthine, guanine, xanthine, DL-methionine, diazotizable amine and UVAC. Positive responses, indicated by zones of growth visible, unless otherwise stated, after 24 hr., were observed as follows:

Yeast 47: adenine, hypoxanthine, UVAC.

Yeast 19: adenine, hypoxanthine, UVAC, diazotizable amine; guanine induced a slight response after 48 hr.

Lactobacillus brevis: plate (*a*) adenine, hypoxanthine, UVAC; plate (*b*) guanine, xanthine, hypoxanthine, UVAC and diazotizable amine; plate (*c*) hypoxanthine, UVAC.

These results showed that hypoxanthine and UVAC had identical biological properties in these tests. Either substituted for adenine in the growth of Yeasts 19 and 47, and for adenine + guanine in the growth of *Lactobacillus brevis*. These facts corroborate the spectrophotometric and chromatographic identification of UVAC as hypoxanthine. Diazotizable amine had activity in lieu of adenine for Yeast 19, and for guanine (but not adenine) for *L. brevis*.

Table 3. *Biological activity of amine concentrates*

Tube tests: growth as Spekker readings after 70 hr. Adenine (col. 6) = 100 μg./ml. (yeasts) or 10 μg./ml. (*L. brevis*); *L. brevis* tested in presence of (*a*) 10 μg. guanine/ml.; (*b*) 10 μg. adenine/ml.; (*c*) no purines; * indicates 10 μg. each of adenine and guanine/ml.

	Amine concentrate (ml./6 ml. basal medium)				
	0	0·02	0·04	0·08	Adenine
	Growth (Spekker reading)				
Yeast 19	0·06	0·44	0·56	0·68	0·99
Yeast 47	0·03	0·18	0·32	0·57	1·07
Lactobacillus brevis (*a*)	0·01	0·01	0·02	0·02	—
Lactobacillus brevis (*b*)	0·01	0·38	0·60	0·85	—
Lactobacillus brevis (*c*)	0·01	0·16	0·31	0·49	0·94*

Table 4. *Antagonism of hypoxanthine by guanine in the growth of* Lactobacillus brevis

Tube tests; growth as Spekker readings after 47 hr.

	Added hypoxanthine (μg./ml.)				
	0	2·5	5·0	10·0	20·0
	Growth (Spekker reading)				
Basal medium C	0·06	0·19	0·30	0·45	0·64
Basal medium C + 10 μg. guanine/ml.	0·07	0·08	0·08	0·09	0·09

Tube tests on unseparated amine concentrate confirmed that it substituted for adenine in the growth of Yeasts 19 and 47, and for guanine or for adenine + guanine in the growth of *Lactobacillus brevis*. Although these responses were expected from the hypoxanthine (UVAC) content of the concentrate, there was an unexpected inability to replace adenine for *L. brevis* (Table 3). The effect of guanine on the growth of *L. brevis* in basal medium C supplemented with hypoxanthine was therefore studied. The results (Table 4) showed that the growth-promoting activity of hypoxanthine was antagonized by guanine.

This antagonism may be compared with that found by Fairley & Loring (1949) to operate for a mutant of Neurospora. The discrepancy that hypoxanthine substituted for adenine in the auxanograms, but not in the tube tests, may be explicable in terms of a concentration gradient operating in favour of the test material in the auxanograms.

Comparison of diazotizable amine with 'carboxamide' and 'carboxamidine'

The properties of synthetic 4-amino-5-imidazolecarboxamide (carboxamide) and the corresponding amidine (carboxamidine), which are recognized as possible bio-precursors of purines, differed from those of diazotizable amine as follows:

(1) The peak absorptions of the coloured substances formed in the Bratton & Marshall reaction differed. Measured approximately by means of the Ilford 601–606 series of filters in the Spekker absorptiometer, the maximum absorption of the coloured solution from diazotizable amine occurred with filter 603, in contrast to filter 604 in the other cases.

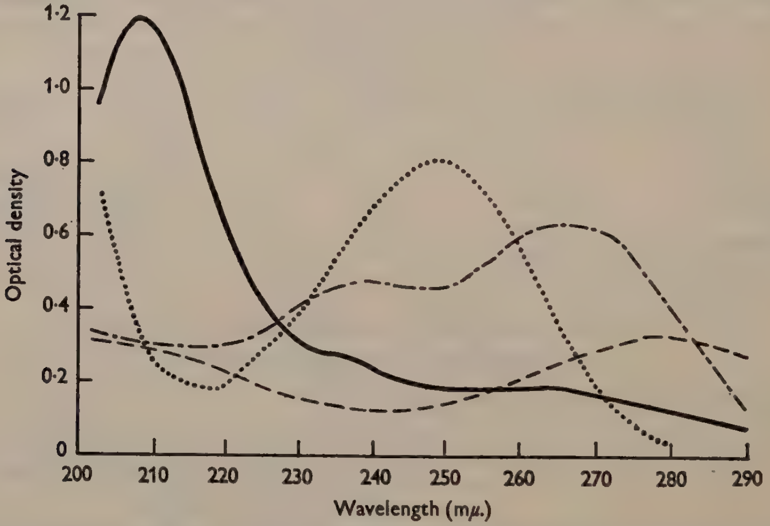


Fig. 1. Ultraviolet absorption curves of solutions in 0·1 N-HCl of diazotizable amine (—); hypoxanthine (. . .); carboxamide(·-·-); carboxamidine (---). Concentrations 10μg./ml., except amine which consisted of 5 ml. of extract derived chromatographically from 5 × 30 μl. samples of amine concentrate.

(2) The ultraviolet absorption curves differed (Fig. 1).

(3) On chromatographing, the R_F values obtained were: amine, 0·36; carboxamidine, 0·43; carboxamide, 0·47. For the chromatographic detection of the two latter compounds, the UV photographic technique was applied, since both substances absorbed appreciably at 257 mμ.

(4) The biological activities of carboxamide and carboxamidine were distinct from those of diazotizable amine. Using small amounts of solid

substances applied to the surface of auxanograms with a platinium needle, it was shown that: neither carboxamide nor carboxamidine possessed activity as adenine substituents for Yeast 47; carboxamidine, but not carboxamide, substituted for adenine in Yeast 19; carboxamidine substituted for adenine, for guanine or for adenine+guanine in *Lactobacillus brevis*, although, in each case, the zone of growth was small compared with that induced by a roughly equal quantity of hypoxanthine. This suggested that carboxamidine was required in relatively high concentration. Carboxamide showed no capacity to substitute for purines in the growth of *L. brevis*.

Effect of amino acids and purines on the accumulation of amine in culture filtrates

Tube tests were used to study the effects of added amino acids and purines on amine formation in an otherwise suitable medium. The results (Table 5) showed that aspartate, threonine and norvaline strongly depressed amine formation, as did norleucine in the early stages. Increased amine formation after 192 hr. in the presence of glutamate, lysine and serine was associated with higher final levels of growth, amine formation in terms of unit dry weight of cells being less than, or equal to, the control. There was some suggestion that, after 96 hr., histidine stimulated amine formation.

Table 5. *Effect of amino acids and purines on amine formation*

Tube tests in basal medium A containing 500 μg. DL-methionine/ml. and 20 $\mu\mu$g. D-biotin/ml. Growth and amine as Spekker readings. Amino acids tested at 500 μg. (as L-isomer), purines at 50 μg./ml.

	Growth		Amine		Amine/unit dry-wt. cells	
	96 hr.	192 hr.	96 hr.	192 hr.		
	Spekker readings					
Basal medium plus						
No additions	0·33	0·39	0·13	0·11	0·66	0·45
Amino acids:						
L-Aspartic acid	0·40	0·41	0·03	0·02	0·12	0·08
L-Glutamic acid	0·47	0·59	0·12	0·20	0·36	0·45
L-Histidine	0·31	0·43	0·13	0·16	0·67	0·56
L-Lysine	0·54	0·64	0·18	0·17	0·47	0·35
DL-Norleucine	0·40	0·59	0·07	0·12	0·27	0·27
DL-Norvaline	0·37	0·54	0·05	0·08	0·21	0·19
DL-Serine	0·37	0·52	0·12	0·13	0·51	0·29
DL-Threonine	0·36	0·43	0·07	0·07	0·32	0·24
Purines:						
No additions	0·50	—	0·25	—	0·72	—
Adenine	0·56	—	0·10	—	0·24	—
Guanine	0·50	—	0·24	—	0·69	—
Hypoxanthine	0·50	—	0·25	—	0·72	—
Uric acid	0·50	—	0·16	—	0·46	—
Xanthine	0·51	—	0·23	—	0·64	—

The effect of adenine in depressing amine formation (Table 5) was reported by Chamberlain *et al.* (1952). Of other purines tested, uric acid exerted a slight depressant effect, but added guanine, xanthine and hypoxanthine did not affect amine formation.

The partial prevention of amine formation by norleucine was probably due to competitive interference with methionine utilization (cf. Porter & Meyers, 1945; Lampen & Jones, 1947; Harding & Shive, 1948). For concentration ratios of DL-norleucine to DL-methionine between 0·5 and 2·0, when the concentration of norleucine was increased fourfold from 250 μg./ml., the concentration of amine formed remained fairly constant. Since Yeast 47 did not require added methionine for growth when *p*-AB was present, presumably then synthesizing its own requirements, the norleucine methionine antagonism should be overcome eventually in favour of methionine, as was found to be the case (Table 5).

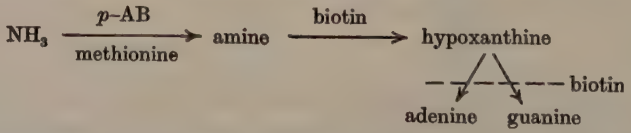
Since no similar competitive interference with methionine utilization was demonstrable for norvaline and aspartate, the prevention of amine accumulation by these amino acids was not explicable on the above grounds.

DISCUSSION

In view of the accumulation of hypoxanthine as well as diazotizable amine in cultures of Yeast 47 grown in defined medium containing methionine and sub-optimal biotin, some modification of the scheme proposed by Chamberlain *et al.* (1952) is necessary. In particular, it seems that the block caused by biotin deficiency occurs after completion of the purine skeleton (as hypoxanthine), the essential systems affected being either or both of those by which hypoxanthine is transformed to adenine and guanine. The results with ***Lactobacillus brevis***, which cannot accomplish the inter-conversion adenine ⇌ guanine, indicate that hypoxanthine (or a derivative) is a common precursor of adenine and guanine. The same may be true for Yeasts 19 and 47, but less evidently so, because both organisms convert adenine to guanine (via hypoxanthine?), only the reverse process being impaired.

As the formation of adenine and guanine from hypoxanthine involves amination, it is interesting to note that Lichstein & Umbreit (1947) reported the deaminases of aspartic acid, serine and threonine to be specifically activated by biotin. In the case of aspartic acid, the reverse (amination) reaction was also activated by biotin. It is possible to explain our biotin-conditioned synthetic block in the light of these findings. Thus if aspartate (and/or threonine) are amino group donors in the amination of hypoxanthine to adenine (and possibly guanine), then failure to synthesize aspartate by amination (a biotin-dependent reaction) would explain hypoxanthine accumulation in our experiments.

Assuming that amine and hypoxanthine are intermediates related by a simple biosynthetic chain, the following scheme is possible:

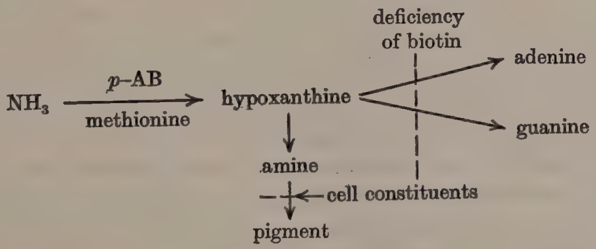


In such a sequence in the cell, the actual substances transformed might be derivatives (e.g. ribosides or ribotides) of the amine and purine molecules indicated. The evidence that diazotizable amine is intermediate in purine biosynthesis is: (*a*) the circumstances under which amine accumulates, the prevention of that accumulation by added adenine and other indirect evidence (see also Chamberlain *et al.* 1952); (*b*) the biological activity of amine as a substituent for adenine (Yeast 19) and guanine (*Lactobacillus brevis*). There are, however, a number of objections to such a scheme. Thus, amine did not substitute for adenine in the auxanograms of Yeast 47, whereas hypoxanthine did. Again, if it is permissible to attempt an interpretation of the sequence of events in *L. brevis* in terms of the scheme, the failure of amine to substitute for adenine + guanine, whereas it will do so for guanine, is not immediately explicable if hypoxanthine (which will substitute for adenine + guanine in *L. brevis*) is intermediate between amine and guanine. A further obstacle is to explain the accumulation of two intermediates in the same chain as a result of biotin deficiency. Possibly biotin is also concerned with the conversion of amine to hypoxanthine, in which case the step must be insensitive to biotin deficiency relative to the processes responsible for utilizing hypoxanthine.

Following from the above, it is interesting that amine appears to lack the complete purine ring structure. The spectrophotometric evidence suggests that the pyrimidine ring is lacking, since amine solutions do not show the peak absorption between 245 and 285 mμ., characteristic of the purines and pyrimidines examined. Carbon-6 of the purines is known to be donated by CO_2 (Sonne, Buchanan & Delluva, 1946) the fixation of which is known, in other contexts, to be associated with biotin-requiring systems. It is thus possible that amine may lack carbon-6 of the purine skeleton.

If biotin is not implicated between amine and hypoxanthine in the above scheme, it is necessary to suppose that this conversion is freely reversible, so that the accumulation of hypoxanthine, resulting from biotin-deficiency, would lead to a parallel accumulation of amine.

If amine is not in the biosynthetic chain which yields hypoxanthine, it could arise by a side reaction from hypoxanthine and, in turn, react with cell constituents to form the red pigment always found in cultures in which amine accumulated:



This hypothesis is favoured because, taken together with the suggested roles of aspartate and threonine as amino group donors for the conversion of hypoxanthine to adenine and guanine, it provides a simple explanation of the prevention of amine accumulation by added aspartate and threonine, the presence of these amino acids permitting normal amination of hypoxanthine, which thus does not accumulate.

The observations of Friedman & Gots (1952) may also be relevant to this explanation. They showed that hypoxanthine and adenine could be reduced to diazotizable amines. Hypoxanthine yielded a mixture of two amines, one of which was carboxamide; the other, and that from adenine, resemble our amine sufficiently in their UV absorption spectra to suggest that they may be related substances.

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Some Aspects of the Amino Acid Metabolism of *Penicillium chrysogenum*

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SUMMARY: Paper chromatographic methods were used to determine the amino acids in unhydrolysed corn steep liquor and to study the uptake and synthesis of amino acids by *Penicillium chrysogenum*. Of the eleven amino acids found in corn steep liquor all were taken up by the organism prior to the main period of penicillin formation. Only traces of ninhydrin-reacting material were present in the later samples of medium.

Glutamine, in addition to the eleven amino acids detected in corn steep liquors, was found in mycelium grown on medium containing corn steep liquor. Mycelium from *Penicillium chrysogenum* grown on medium containing inorganic nitrogen as sole nitrogen source showed the presence of the same twelve amino acids.

The gross nitrogen metabolism of various strains of *Penicillium* spp. used in the production of penicillin has been described previously by Koffler, Emerson, Perlman & Burris (1945) and by Stefaniak, Gailey, Jarvis & Johnson (1946), but less information is available on the detailed amino acid utilization by the organism in deep culture penicillin fermentations. This paper describes experiments carried out to determine the free amino acids present in corn steep liquor, to confirm their presence in sterilized corn steep liquor medium and to study their individual uptake by *P. chrysogenum* during submerged culture. The synthesis of amino acids from chemically defined medium under similar conditions was also studied.

METHODS

Fermentation conditions. Experiments involving the uptake of amino acids were carried out in lactose + corn steep liquor medium (Rolinson & Lumb, 1953) in 3 l. laboratory scale fermenters of the type described by Lumb & Fawcett (1951). Chemically defined medium in which ammonium nitrogen replaced that of the corn steep liquor on an equivalent total nitrogen basis was used to determine the free amino acids synthesized by the organism under submerged culture conditions in shaken flasks (Lumb & Fawcett, 1951). All fermentations were carried out in duplicate at 26° with a non-pigment forming strain derived from *Penicillium chrysogenum* Q-176.

Preparation of samples for chromatography. The mycelium was filtered off under suction on a kieselguhr pad and the clarified culture filtrate was retained for chromatographic analysis. The mycelium was washed 3 times with sodium chloride solution (0·9 %, w/v) to remove traces of medium and was then freeze-dried. This dried mycelium was ground in a mortar to break the cell walls and 0·2 g. was extracted with 5 ml. ethanol (80 %, v/v, in water) at room temperature for 48 hr., and then filtered. Corn steep liquor was diluted with 3 times its volume of water to facilitate application to the paper for chromatography. The various clarified medium samples were de-salted by electrodialysis before chromatography.

Chromatographic technique. The separation of the individual amino acids was carried out by ascending two-dimensional paper chromatography using Whatman no. 4 paper (28 × 28 cm.) and phenol/water/formic acid mixture (Smith & Agiza, 1951) in $NH_3 + HCN$ atmosphere and acetone/aqueous urea mixture (Bentley & Whitehead, 1950) as first and second solvent systems respectively. Equal volumes of similar samples were applied to separate papers with an Agla micrometer syringe so that the different chromatograms would be comparative. After development with the first solvent the chromatograms were dried overnight at room temperature and after the second solvent mixture for 2 hr. at room temperature. The spots were then detected by spraying with 0·1 % (w/v) ninhydrin in butanol and heating in an oven at 90° for 15 min. The unknown spots were compared with those from single known amino acids chromatographed under the same conditions. Chemical spot tests (Block & Bolling, 1951; Bolling, Sober & Block, 1949) supplemented this method of identification in the case of tryptophan, arginine and histidine.

RESULTS

From unhydrolysed corn steep liquor eleven ninhydrin-positive spots were detected which corresponded to aspartic acid, glutamic acid, glycine and/or serine, alanine, ornithine and/or lysine, tryptophan, proline, valine, leucine, arginine and histidine. Table 1 indicates semi-quantitatively the amounts of each. Many batches of corn steep liquor from different sources showed essentially the same composition. Leucine, isoleucine, valine and glutamic acid had previously been identified in unhydrolysed corn steep liquor by microbiological assay by Cardinal & Hedrick (1948).

Uninoculated medium after sterilization, culture filtrates and mycelium were then examined for amino acids at the beginning of the fermentation and and at 24 hr. intervals (Table 1). All the amino acids originally detected in corn steep liquor were found in the medium after sterilization and at the beginning of the fermentation. All of them were taken up by the organism during fermentation and only traces were present in the 48 hr. and later samples of culture fluid. This contrasts with the situation found with some bacteria where amino acid uptake is strongly selective (Proom & Woiwod, 1949). Traces of two new ninhydrin-positive substances were found in the medium during the course of the fermentation; one was identified as glutamine. The other spot, as yet unidentified, had R_F values of 0·90 and 0·33 in the first and second solvent systems respectively, and thus lay near arginine.

The amino acid content of mycelium grown on corn steep liquor medium was next investigated. All the eleven amino acids found in corn steep liquor were found in the mycelium at some stage of the fermentation, and in general their concentration was at a maximum at 48 hr. and decreased slowly during the remainder of the fermentation. Glutamine was also detected in mycelium of 48 hr. and older but no other ninhydrin-positive material was found (Table 1). Thus the striking fall in the amino acid concentration in the culture fluid at 48 hr. corresponds to an accumulation within the mycelium; it is after this that the main phase of penicillin production takes place, lending support to previous findings by Wolf (1948), and by Narasimha Rao & Venkataraman (1952) that these accumulated amino acids within the mycelium are intimately concerned with the formation of penicillin.

Table 1. *Amino acids in corn steep liquor and in medium and mycelium during fermentation in lactose-corn steep liquor medium*

Amino acid	Corn steep liquor	Culture fluid Time of sampling (hr.) 0	24	48	72	96	120	Mycelium Time of sampling (hr.) 24	48	72	96	120
spartic acid	++	++	++	0	+	+	0	+	++	++	+	+
lutamic acid	++	++	++	0	0	0	0	++	++	++	++	+
lycine and/or serine	+++	+++	+++	+	+	+	+	+++	+++	+++	++	++
lanine	+++	+++	+++	0	+	+	+	+++	+++	+++	+++	++
lutamine	0	0	0	0	0	0	+	0	+	++	++	++
rnithine and/or lysine	++	++	++	0	0	0	0	0	+	0	0	0
ryptophan	++	++	++	0	0	0	0	0	0	++	++	0
roline	++	++	0	0	0	0	0	0	+	0	0	0
aline	+++	+++	+++	0	0	0	0	++	+	+	+	0
eucine	+++	+++	+++	+	+	+	+	++	++	+	+	+
rginine	+	+	+	0	0	0	0	0	+	0	0	0
istidine	+	+	+	0	0	0	0	0	+	0	0	0
nidentified spot	0	0	+	+	+	+	+	0	0	0	0	0

+++, large well-defined spot; ++, small well-defined spot; +, trace; 0, not detected.

Table 2 shows the results of similar experiments on fermentations in chemically defined medium where ammonium and nitrate were the sole sources of nitrogen. Chromatography of extracts from mycelium grown for 12 days showed the presence of the same twelve amino acids previously found in mycelium grown on corn steep liquor + lactose medium. As in the latter the glutamine, ornithine and/or lysine, tryptophan, arginine and histidine concentrations were very low, and the amino acid pattern is very similar. Thus the fungus readily synthesized its complement of amino acids from inorganic nitrogen. The medium at the end of the fermentation showed the presence of three spots corresponding to alanine, glycine and/or serine and leucine. Thus some excretion or autolysis of the mycelium had taken place.

The mycelium from corn steep liquor and from chemically defined medium was autolysed at pH 5·3 for 72 hr. at 36° under a layer of toluene and the autolysates examined as before. (Table 2). The only changes detected were the disappearance of glutamine and some loss of tryptophan.

An interesting feature of all these analyses is in the similarity of the free amino acids in the corn steep liquor and those liberated from the mycelium after hydrolysis.

Table 2. *A comparison of the amino acids found in fresh and autolysed mycelium grown in corn steep liquor medium and chemically defined medium*

	Mycelium grown on medium containing corn steep liquor		Mycelium grown on medium containing inorganic nitrogen		Amino acids in chemically-defined medium after fermentation
Amino Acid	Fresh	Autolysed	Fresh	Autolysed	
Aspartic acid	++	++	++	++	0
Glutamic acid	++	+++	++	++	0
Glycine and/or serine	+++	+++	+++	+++	+
Alanine	+++	+++	+++	+++	++
Glutamine	+	0	+	0	0
Ornithine and/or lysine	+	++	+	++	0
Tryptophan	++	+	+	0	0
Proline	+	++	++	++	0
Valine	+	+++	+++	+++	0
Leucine	++	+++	+++	+++	++
Arginine	+	++	+	+	0
Histidine	+	++	+	+	0

+++, large well-defined spot; ++, small well-defined spot; +, trace; 0, not detected.

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Cryptococcus terreus n.sp., from Soil in New Zealand

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SUMMARY: A new species of *Cryptococcus* has been isolated from six of eight soil samples taken in the province of Otago, New Zealand. It is distinguished from other species of the genus by its ability to utilize glucose, maltose, lactose, galactose and potassium nitrate, but not sucrose.

In the course of a search for pathogenic yeasts in New Zealand soils, eight soils from the province of Otago were sampled for their normal yeast flora. They were cultured on Sabouraud agar of pH 4 and incubated at room temperature. Twenty-four strains of yeasts from each soil sample were subcultured and identified. Six of the soils yielded a hitherto undescribed species of *Cryptococcus*, which comprised thirty-eight of the total 192 isolates. It is proposed to name this yeast *Cryptococcus terreus.*

Cryptococcus terreus sp.nov.

Cellulae in culturis novae rotundae aut subovoidae (7·5–5·0) × (7·0–3·0) μ. Cellulae circumdatae capsula mucosa. Cultura nova in agaro Sabouraudi albida, mucosa. Cultura (post unum mensem) flavifusca, tenax. Sedimentum et anulus formantur. Pseudomycelium nullum.

Fermentatio nulla. Glucosum, maltosum, galactosum, lactosum assimilantur. Sucrosum non assimilatur. Nitras kalicus assimilatur.

Cell from young cultures globose to slightly oval with mucous capsules. Cell size (disregarding capsules) (7·5–5·0) × (7·0–3·0) μ. Growth on solid media at first cream and mucoid, later tan with a tough surface skin. A ring and sediment but no pellicle in broth. No mycelium observed.

Fermentation:* absent.

Sugar assimilation:*

Glucose +	Lactose +
Sucrose −	Galactose +
Maltose +	

Assimilation of potassium nitrate:* positive.

A starch-like substance is elaborated on the defined medium of Mager & Aschner (1947).

I am indebted to Miss A. v. d. Hoven v. Genderen of the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, for the following additional information: splitting of arbutin, strongly positive; ethanol as sole source of carbon, no growth; splitting of fat, negative.

* For details see Lodder & Kreger-Van Rij, 1952.

COMMENTS

No ascospores were seen when this yeast was subcultured upon carrot plugs, Gorodkowa agar or Starkey's medium (1946). In the absence of a perfect stage, ballistospores, or mycelium, the species was placed without hesitation in the genus *Cryptococcus*. The mucous capsules about the cells were well marked (see Fig. 1), and production of starch-like substance was rapid and pronounced.

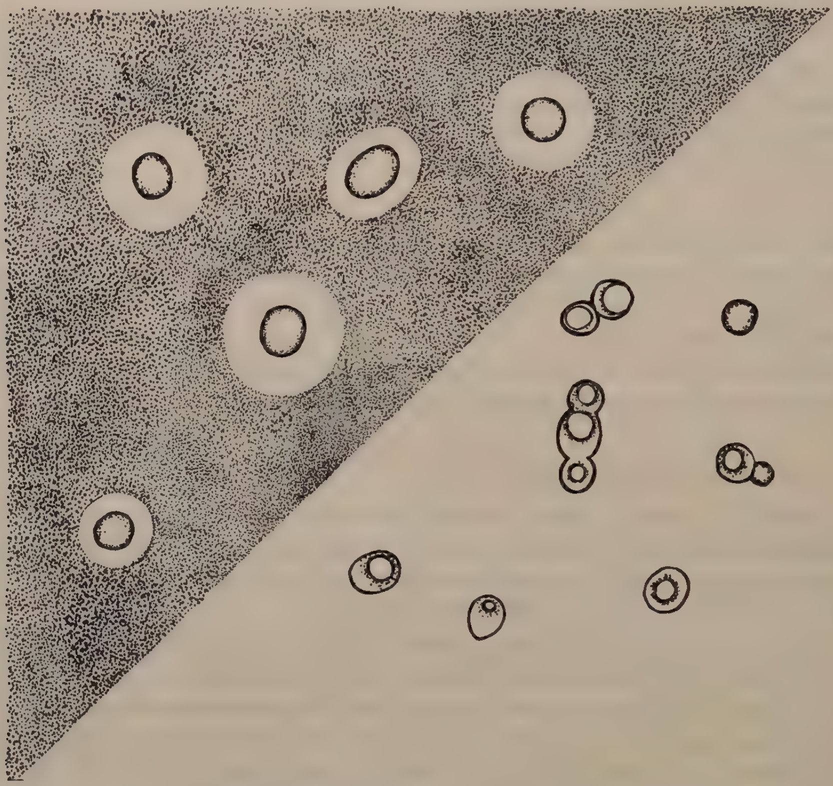

Fig. 1. Cells of *Cryptococcus terreus*. × 1000.

Cryptococcus terreus closely resembles *C. albidus* on all points but the ability to utilize sucrose. It appears that its distribution may be similar also, for *C. albidus* was found in large numbers in the soils sampled, making up sixty-four of the 192 isolates of the survey mentioned above. *C. terreus* has not yet been found in any site but soil. A survey of aerial yeasts has been made in this laboratory during the past year, and *C. terreus* has not been encountered amongst the several hundred isolates so far made.

Cultures of the type strain of *Cryptococcus terreus* (isolated from soil from a garden in the suburb of Roslyn, Dunedin, in July 1953) have been deposited with the Yeast Division, Centraalbureau voor Schimmelcultures, Delft; The

Brewing Industry Research Foundation, Nutfield, Surrey, England; and the American Type Culture Collection, 2029, M. Street, N.W., Washington 6, D.C.

I am indebted to Miss A. v. d. Hoven v. Genderen of the Centraalbureau voor Schimmelcultures for confirmation of the validity of this species.

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Factors Promoting Cell Division in a 'Soft' Mycelial Type of Nocardia: *Nocardia turbata* n.sp.

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SUMMARY: Various methods of preparing microcultures with liquids (hanging-drop) and solids (collodion, cellophan, agar) were compared with respect to the mode of growth of the test organism on a complex nutrient substrate. Cell division was promoted in general by lower temperatures, by ageing, by lack of humidity in solid cultures, by the action of surface tension operating in the more convex droplets, and by the addition of surface-active agents to liquid media. By varying the carbon sources in chemically defined media it was found that starch, glycogen, and to some extent glucose, favoured filamentation, i.e. retarded or inhibited cell division, in comparison with a considerable variety of other sugars. Of the nitrogenous sources, nitrate resulted in relatively poor growth mainly composed of mycelial aggregates that later exhibited cell division to the extreme limits; ammonium salts yielded more profuse filamentous growth with less subdivision; certain single amino acids induced a more uniform development of short rods; while casein hydrolysate commonly produced a variety of cell elements showing subdivision in all stages. Single cell isolates were used throughout, and the mode of growth was studied by means of phase-contrast and electron microscopy.

A cultural description of the test organism, *Nocardia turbata*, n.sp. is given at the end of the paper.

The indispensable criterion of the actinomycetes as a family is the formation of a branching mycelium. The outstanding character of the genus *Nocardia* is the fragmentation of this initial mycelium into short or long elements: branching, filamentous, bacillary or coccoid. The simplest way of subdividing this genus is in accordance with: (*a*) the rapidity or otherwise with which spontaneous fragmentation takes place; (*b*) the degree, partial or complete, to which cell division is carried on with ensuing breaking-up of the mycelium. This is, in fact, the basis of the morphological classifications of Ørskov (1923) and Jensen (1931), and of the derivative scheme proposed by Waksman & Henrici (1943) and adopted in the 6th edition of *Bergey's Manual* (1948).

A 'soft' mycelial type of nocardia is, therefore, one in which cell division proceeds more or less rapidly and to the extreme limits. As a direct consequence of this fragmentation, such a strain exhibits in liquid cultures some degree of turbidity (as against the characteristic clarity shown by actinomycetes with structurally intact mycelium), while on solid substrates massive sowings result in smooth readily emulsifiable growth which entirely resembles that of many ordinary bacteria. The fragmented cells themselves often retain V- and Y-forms similar to those of corynebacteria. Indeed, it is because of the systematically interesting position—at the borderline between eubacteria and actinomycetes—that it is of value to study the ways in which cell division takes place within the transient chains and branching filaments of these short-lived mycelial aggregates.

ORGANISM

The test organism used here has several interesting features:

(1) On most suitable solid media single cells develop into small mycelia showing true branching, which are entirely characteristic of actinomycetes in general (Pl. 1, fig. 1). These fragment rapidly on poor substrates (Pl. 1, fig. 2), while on rich complex media such as nutrient agar the peripheral filaments of well-spaced colonies may be seen branching and radiating into the depths of the agar even after 2–4 weeks of growth at room temperature. It is therefore not possible to doubt that this organism is an actinomycete, as is sometimes possible with very 'soft' species of *Nocardia.* On the other hand, the cell mass of even such aged single colonies is of a pasty texture, and can readily be removed from the medium and the component cells smoothly dispersed in water. Moreover, by the use of soft agar and massive inocula, moist smeary growths can be produced which look entirely bacterial, while in liquid cultures turbidity is the rule.

(2) The phenomenon of fragmentation is made evident in more than one way. (*a*) By the transformation of almost the entire central mass of the growth on solid substrates into bacillary cells of varying lengths (Pl. 1, figs. 2, 4), which may show 'slipping branching' (Pl. 2, fig. 11), giving rise to 'palisade formation' (Pl. 2, fig. 15); or into coccoid cells which in certain liquid or semi-solid nutrients may cohere almost like staphylococci (Pl. 1, fig. 5); or into a mixture of rods and 'cocci' (Pl. 1, fig. 3; Pl. 2, fig. 17). (*b*) By the varying degrees of motility shown under different circumstances by many of these polymorphous cells. So far motility has been reported among the actinomycetes only within the genus *Nocardia* (Topping, 1937; Ørskov, 1938). The organism studied here is one (no. 27) isolated by Dr Ørskov from Danish soil, and kindly sent to me by him. It produces an insoluble yellow-green pigment on protein-containing media, and is clearly allied to the other yellow- or green-pigmented 'soft' mycelial forms such as *N. flava*, *N. viridis* and *N. citrea* (see also Jensen, 1953). It agrees with these non-paraffin-utilizing species in having non-acid-fast and in general weakly refractile cells. It differs from them in the production of 'cocci', which are often more highly refractile than the filamentous cells, and above all by the property of motility. These are constitutional differences, which appear to merit separation by species. The specific name *turbata* (Lat. = restless, troubled) is proposed, and a cultural description of the organism is given under the name *Nocardia turbata* n.sp.

In the course of these studies it was soon realized that while in all circumstances cell division constantly precedes motility, it was not necessarily followed by motility. Cell division accompanied by fragmentation could and frequently did take place without any subsequent motility on the part of any of the variously shaped cell elements. The conditions which favoured motility could therefore to some extent be considered separately; this will be done in a future communication. In the present work the emphasis has been placed upon cell division, while noting the presence or absence of motility.

In view of the morphological diversity to be found within this species, twelve single-cell isolates were prepared by means of the de Fonbrune micromanipulator (for details see Erikson & Masson, 1954). Stock cultures of these pure line strains were maintained on nutrient (peptone+Lab Lemco+Marmite) agar at room temperature. Aqueous suspensions of washed cells from such nutrient agar slopes were employed as inocula, and the cells of two different isolates were used in every experiment.

METHODS

Liquid media. The growth of *Nocardia turbata* in a variety of media was studied in small volumes (2 ml. in 4 in. × $\frac{1}{2}$ in. test-tubes), which gave good aeration favouring profuse growth. At room temperature (18–23°), the optimum for the demonstration of motility and suitable for growth in general, there was little evaporation in the course of 1–2 weeks. A fairly heavy inoculum (5×10^6 cells/ml.) was required to establish characteristic growth in the various media within 2–6 days, the peak period of growth. This inoculum was obtained by washing the growth from a 2-day nutrient agar slope, resuspending it in 5 ml. water, spinning lightly to throw down the bulk of the mycelial fragments, and matching the suspension, containing mainly rod and coccoid elements, to a given arbitrary standard for each experiment; 0·1 ml. of the suspension was added to each tube.

The media used can be classified according to their composition as follows:

Media containing nitrogen as nitrate. A basal medium of Czapek mineral salts with sodium nitrate, to which was added: 0·5 % (w/v) of fermentable sugars, occasionally 0·1 % yeast extract (Yeastrel), or 0·025 % of various surface active agents such as Tween 80 or Nonex 139.

Media containing nitrogen as ammonium salts. The basal Czapek mineral salt medium with ammonium phosphate, ammonium chloride, or ammonium sulphate in place of the sodium nitrate. Sugars added in the same proportion as above.

Media containing nitrogen as single amino acids. The basal Czapek mineral salt medium with a single amino acid (0·1 %, w/v) in place of the sodium nitrate. Sugars added in the same proportion as above.

Media containing nitrogen as a mixture of amino acids. The basal Czapek mineral salt medium with 0·1 % (w/v) of an acid hydrolysate of casein (vitamin-free). Two preparations were used; one made by Allen and Hanburys Ltd., the other by Ashe Products, Ltd. Again sugars were added in the same proportion as above. In all instances the sugars were sterilized separately, added aseptically to the sterile basal media, and then briefly steamed.

Media containing nitrogen in more complex forms. These included standard nutrient broth of the same composition as the nutrient agar on which the stock cultures were maintained; and with the following additions: 5 % tryptic digest of casein; 5 % heart broth; 5 % brain-heart infusion (Difco). Occasionally surface active agents were added to the plain broth in the proportions noted above.

The pH value of all media was adjusted to 7·2.

Micro hanging-drop cultures. These were prepared according to the method of Webley (1953), with the modification that the spraying vessel was connected by means of a ground glass joint to a 'Quickfit' Pyrex tube 8 × ½ in., which was then covered by a metal cap with a hole, ⅛ in. diameter, drilled in the centre. The coverslip to be sprayed was inverted over this cap. All the components of this system can be sterilized separately and assembled quickly. In their passage through the relatively narrow lumen of the tubing, the droplets did not evaporate so that there was no need for a special moist chamber. Otherwise the procedure throughout was as described by Webley. The slides were examined daily by the ordinary light microscope.

Microcultures using solid media. The same dilute suspensions used for spraying the hanging-drop cultures, employing media of all the classes mentioned, were examined for the mode of growth of the cells in an agar film. In such cases the sprayed and dried slips were inverted over a plain slide bearing a drop of molten 1 % agar + the basal medium, gentle pressure exerted to expel air bubbles, and the growing preparation (kept in a moist chamber) examined at daily intervals by phase-contrast microscopy. In other cases a small block of solidified medium under test was placed on the slide, which was sprayed directly with a dilute aqueous suspension of cells, or inoculated with a loop in the manner of Ørskov (1923). Where the simultaneous comparative observation of a number of preparations was required, the agar plaque method of Turner-Graff (1952) was found useful. A modification was made as regards inoculation in that the Petri dish containing the separate agar plaques was inverted over the spray, and each plaque sprayed in turn. Individual colonial development could be followed in these cases.

In addition, there was always available material of varying ages resulting from the sowing of nutrient agar slopes for suspensions, and the plating out of these suspensions as a check for purity.

Microcultures on cellophan and collodion membranes. To the advantages of clarity of observation and freedom from contaminating medium noted by many workers when a sheet of sterile cellophan is placed between medium and organism, there must be added, in the case of the actinomycetes, the further benefits that the growths in general tend to be flattened out with a greater proportion than usual of the cells developing in the same plane, while individual cells show a higher degree of elongation. These observations were first made by Lentze (1938) in connexion with the anaerobic actinomycetes, and have been repeatedly confirmed by the author for all groups of actinomycetes (Erikson, 1940, 1947, 1949, 1952).

Duplicates of the microcultures on agar plaques were prepared by placing circles of sterile cellophan over the plaques, incubating overnight at 30° to test for sterility, and then spraying the cellophan surface of each plaque in turn. The resultant growth was used for both comparative observation and as a source of clean cells for electron micrographs (see Pl. 2, figs. 14–16). When pictures of cells actually growing on a collodion membrane were wanted, it was found helpful to support the membrane on small squares of cellophan and so interpose two layers between substrate and growing cells. This was done by placing the cellophan square on the S-shaped end of a bent glass rod and gently inserting it under the water on whose surface the collodion membrane had been poured in the usual way. When the rough margins of the membrane had been trimmed away with a needle, the collodion + cellophan square could be treated as a single unit and sterilized by steaming. It was then placed over a hollow ground slide, the cavity of which had been filled with a suitable solid medium, incubated in a moist chamber to test for sterility, and sprayed as above. After growth the complete unit, collodion + cellophan, was picked off the medium, fixed in formalin vapour, and floated on clean water, where it was a simple matter to prise away the supporting cellophan and collect the membrane on a grid. This method gave very clean pictures (see Pl. 2, figs. 10, 11 and 13).

In certain instances a chart of the specimens on the dried grid was made on squared paper, plotting the cells in certain marked areas of the grid as seen through the 4 mm. objective of the light microscope. Such areas could then be found in the electron microscope, and, after micrographs of suitable cells in that area had been taken, they could be compared with the appearance of the cells in the light microscope. For permanent reference both collodion and cellophan membranes bearing fixed cells could be treated as sections, floated on warm water to smooth out wrinkles, and mounted on slides smeared with 1 % gelatin.

RESULTS

It will be convenient to consider first those factors of a physical or general nature that influence the cell division of *Nocardia turbata*. Since such factors were generally operative regardless of the nature of the medium, their activity will be examined mainly in cultures on rich complex media, unless noted to the contrary. The consistently abundant growth on the stock nutrient agar or broth allowed a ready detection of these factors.

Effect of temperature

Lower temperatures induced early cell division and fragmentation, and were essential for the demonstration of motility. Thus, Pl. 1, fig. 1, represents the characteristic filamentous mycelial growth resulting from 24 hr. of incubation at 30°, while duplicate sets incubated for the same time at 20° constantly showed a greater or lesser degree of cell division and fragmentation (Pl. 1, fig. 2). Although the growth in Pl. 1, fig. 2, is an older growth on a poorer medium (soil extract agar), the way in which the subdivision of the internal cell-mass of the microcolony has proceeded is entirely similar to that customary in a 24 hr. microculture on nutrient agar at 20°. In the latter case it is often difficult to obtain such a clear stationary picture when there is any film of moisture round the cells, since considerable numbers of the shorter cells exhibit varying degrees of motility as soon as a coverslip is placed over the growth.

Effect of humidity

Other things being equal, a saturated aqueous environment promoted early subdivision of the cells into rods and filaments of varying lengths which tended to maintain their dimensions on multiplication (Pl. 1, fig. 3), while under arid conditions cell division proceeded to the extreme limits (Pl. 1, fig. 4). This difference was frequently observed between dry and moist agar mass cultures on the one hand; or in more detail where one set of cellophan microcultures was kept saturated for 10–14 days at room temperature, while the duplicates (in similar close-fitting slide boxes, lined with moistened wadding) were allowed to stand untended until the end of the experiment. A further factor is probably introduced in the latter instance as the result of the decreasing supply of nutrients diffusing through the drying membrane.

Effect of surface tension

As mentioned in Methods, the generally marked elongation of the cells is one of the main advantages of employing cellophan in microcultures. It seems probable that once a cell has begun to grow within the film of moisture surrounding a membrane and in a plane parallel to it, there is a strong opposing force against any upwards movement on the part of the cell away from the membrane. This can readily be demonstrated in micromanipulative practice by attempting to remove a relatively long filamentous cell away from a moistened cellophan surface by means of a microhook or loop. The resistance will be found to be considerable.

On the surface or in the immediate subsurface of solid gels such as 2% agar, *Nocardia turbata* develops a peripheral margin of comparatively long filaments or chains of cells, which may persist for weeks unfragmented, although the cell divisions are frequently clear. In slowly liquefying gelatin cultures the cells quickly divide into short rods and almost spherical coccoid forms, frequently with thickened cell walls (Pl. 1, fig. 5).

In the very small droplets of liquid broth delivered either by the spraying technique or by means of a micromanipulator, the detailed picture varied somewhat. Thus, it was a common occurrence to find that a single cell would elongate to a slight degree only, then divide by binary fission; the daughter cells would perhaps divide again or exhibit motility first, and not until four or five rods were present in the droplet would elongation and branching take place to produce a minute mycelium. In general, the shallower and smaller the drop the more filamentous the growth; so that in more convex droplets on the same slide cell division usually proceeded further with the production of a larger number of short cell elements. On the other hand, when one cell only was placed in a relatively large volume of broth (0·5 ml. in a small tube) and the culture grown without shaking, a characteristic actinomycete type of growth resulted, namely a coherent mycelial complex of cells in a clear medium (see Erikson & Masson, 1954). Fragmentation followed at least a day later, and in these circumstances it was clear that elongation and branching to some extent preceded or kept abreast of cell division.

When surface-active agents such as Tween 80 were added to liquid cultures (2 ml. volume), the chief result was a rapidly increased rate of cell division with a resultant predominance of small coccoid forms. Such very short elements rarely displayed more than Brownian motion. Larger pyriform cells, similar to that portrayed at the end of a filamentous branch in Pl. 2, fig. 13, were frequently found, especially in older cultures.

Effect of ageing of cultures

The unknown changes which take place in complex media like nutrient broth as the result of profuse nocardial growth are probably attributable to both physical and chemical processes. They will include depletion and alteration of nutrients, altered pH value, appearance of metabolic and autolytic products. Seitz filtrates of old culture fluids, and of aqueous suspensions of old cells which had been allowed to autolyse for 2–3 weeks at 37°, were added to young cultures in varying proportions, but failed to induce any marked acceleration of cell division. Nor were positive results obtained by altering the pH value within the range permissible to growth. In general, ageing was always accompanied by subdivision into shorter cells, but the time of onset and degree of subdivision depended upon the nature of the medium and other environmental factors.

In the following two sections chemical factors capable of influencing growth in the simpler media are considered.

Effect of nature of carbon compound in the media

The addition of polysaccharides such as starch and glycogen constantly favoured the production of a filamentous mycelium and inhibited to a considerable extent the process of cell division and onset of fragmentation. Thus, in a glycogen + nitrate liquid medium a granular bottom growth occurred which left the supernatant medium clear, the growth consisting almost entirely of comparatively long filamentous cells, all non-motile.

Among the simple sugars glucose was the only one which consistently induced a similar persistently filamentous growth. In a series of experiments in which various sugars were added to a basal ammonium phosphate medium, it was found that the proportion of long cells obtaining after 2 weeks of incubation was roughly comparable with glucose, starch, or glycogen and much higher than with galactose, lactose, fructose, sucrose, mannose, sorbose, arabinose, melezitose, xylose, raffinose, rhamnose, mannitol, dulcitol, sorbitol or glycerol.

Although glucose was readily fermented (lowering the pH value to 5·0 within 7 days), this was in varying degrees also the case with galactose, lactose, maltose, sucrose, xylose, arabinose, glycerol, or starch, especially in a casein hydrolysate basal medium. A comparative examination of the growths obtained with different carbon sources added to the four defined basal media made it clear that the pH value alone was not a decisive factor in the acceleration or retardation of cell division. Nor did there seem to be any relationship between pH value and the occurrence of motility, which was evident and sustained in presence of galactose, but not with mannose, fructose, or raffinose.

Effect of nitrogen source used

In general, the use of the media containing nitrate as nitrogen source resulted in a relatively small amount of growth which consisted initially of a high proportion of mycelial complexes later fragmenting into very short rods and coccoid cells that were non-motile (Pl. 1, fig. 4). When surface-active agents (e.g. Nonex 139) were added, the coccoid nature of the cell elements was greatly emphasized.

The media containing ammonium ion as nitrogen source supported a more profuse growth in which the mycelial phase was very transient and the predominant cell elements were rods of varying lengths. Long rods, often showing a subdivision into four or eight component cells (Pl. 1, fig. 9), were a feature of ammonium sulphate cultures. Paired cells, frequently motile, were very common in the ammonium chloride and ammonium phosphate cultures. On the whole, branching was less evident in the ammonium than in the nitrate media.

The tendency to grow and multiply almost exclusively in the form of short bacterium-like rods, with a partial or even complete submergence of the branched mycelial phase, became more marked in the case of media which contained those single amino acids that were capable of supporting good growth. Thus, Pl. 1, fig. 6, illustrates the short regular rods, often actively motile, in a 4-day culture in galactose + glutamate medium. Similar more or less uniform rods were obtained with glutamic acid, proline, glycine, alanine, arginine or asparagine as sole nitrogen source; very much shorter cells and generally poorer growth occurred with cystine, valine, methionine, leucine, tryptophan or aspartic acid; very slight or no growth occurred with lysine. The addition of glucose to those amino acids which supported only fair growth, e.g. aspartic acid, resulted usually in the production of a fair proportion of long filaments which showed little or no branching and which were non-motile.

A mixture of short and long rods and filaments, but exhibiting on the whole very little branching (Pl. 1, fig. 3), was yielded by growth in the complex of amino acids of an acid hydrolysate of casein. The addition of glucose tended to prolong the mycelial filamentous stage and to retard cell division, as compared with parallel sets of cultures containing galactose. With a massive inoculum galactose + casein hydrolysate cultures maintained a more or less stable equilibrium, with a predominance of rod forms, many of them motile, for relatively long periods of time, e.g. 3–4 weeks in a stoppered 100 ml. bottle. With the complex nitrogenous media growth was so abundant and the sequence of events so telescoped that it was possible to find almost all the stages recently described in a single 24 hr. culture on nutrient agar (see Pl. 2, fig. 10–18).

DISCUSSION

The dense opaque bodies noted by many workers as occurring in species of *Nocardia* were found in *N. turbata* in similar positions: at the poles of cells (Pl. 1, fig. 8; Pl. 2, fig. 18), and more or less evenly distributed along the course of fragmenting filaments (Pl. 2, figs. 10, 11, 15 and 17). Like McClung (1950), to whose paper reference should be made for a clear and critical account of these phenomena, I have been unable to determine the role, if any, played by such bodies in cell division. With the best optical system in the phase-contrast microscope, living cells about to divide appeared as a rule as dense as the two filaments shown in the electron micrograph of Pl. 2, fig. 14.

Confirmation was obtained of the persistence of fine extensions of the cell wall after the separation of the cytoplasmic contents (Pl. 2, figs. 11, 15 and 16). This was the case whether the cells were photographed *in situ* on the membrane on which they were grown (Pl. 2, fig. 11), or whether they were removed by washing from the substrate on which they had grown (Pl. 2, fig. 16). On the other hand, there appear to be clear-cut cross walls in Pl. 2, fig. 10, which is another portion of the specimen shown in Pl. 2, figs. 11 and 13. However, when the cells at the growing tips of the filaments in these three pictures are carefully examined, the inequalities in diameter reveal a preliminary separation of the cytoplasmic contents, and it seems probable, as suggested by McClung, that the rupture of the outer wall occurs subsequently. The delicacy and elasticity of the outer cell wall was very evident throughout the studies of the living forms. In Pl. 1, fig. 3, for example, can be seen several spirally twisted cells which had just been moving; while in the lower chain of cells in Pl. 1, fig. 9, only two of the cells are in focus because of the slight spiral motion.

In concluding the account of the plasticity of cell forms in *Nocardia turbata*, their variety may be recapitulated as follows: on germinating short cells elongate, then according to environmental conditions (medium composition, temperature, liquid or solid culture, air supply, etc.) they may divide or branch, spin, rotate on their axes, swim, or form small or large complexes, which in turn may cohere for some time or fragment almost immediately; the branches may be motionless or actively motile in short cell, rod, filament, or 2-, 3- or 5-branched stages; and any one of these cell forms can give rise to the normal development again, but some factors (physical or chemical) favour, extend, accelerate, or retard one phase at the expense of the others.

Diagnostic description of Nocardia turbata *n.sp.*

Morphologically, the organism is a typical Gram-positive actinomycete, producing a small mycelium composed of slender filaments (0·1 μ average diameter), which fragments into rods and coccoid cells. Many cells are motile under appropriate conditions; filamentous and rod-shaped cells weakly refractile; cocci in older cultures more strongly refractile. Non-acid-fast.

Aerobic. Optimum temperature 20–30°. Good growth on media such as nutrient (peptone + Marmite or Lab Lemco) agar. Colonies small, 0·1–2·0 mm. diameter, taking 3–4 weeks to grow to full size. Growth initially colourless, producing a yellow-green pigment in 2–4 days on thickly sown slopes of nutrient agar. Pigment production favoured by a free air supply, suppressed by acid pH values. Individual colonies may retain filamentous margins for prolonged periods. Young broth cultures turbid, with subsequent sedimentation of the cells, pellicle formation, and eventual clarity of the medium (4–6 weeks).

Acid without gas produced within 7 days from glucose, sucrose, maltose, lactose, galactose, xylose, arabinose, glycerol, starch; not from mannitol, raffinose, rhamnose, sorbitol, dulcitol (using a casein hydrolysate basal medium). Nitrate nitrogen utilized. Gelatin not hydrolysed except in the presence of peptone, and then only slowly (3–6 weeks). Paraffin not a suitable source of energy.

Habitat. Probably soil.

The type culture is maintained in the Collection of the State Serum Institute, Copenhagen, Denmark.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Filamentous branched mycelial microcolonies, 1 day at 30°, on cellophan over nutrient agar. Phase contrast. (× 300.)

Fig. 2. Fragmentation of central cell-mass of mycelia, 4 days at 20°, on cellophan over soil extract agar. Phase contrast. (× 300.)

Fig. 3. Long rods with little branching, several spirally twisted; shorter rods and coccoid cells, the latter mostly out of focus; 8 days at 20° in liquid casein hydrolysate medium. Phase contrast. (× 1080.)

Fig. 4. Complete fragmentation into short cell elements, 10 days on cellophan over glucose Czapek agar; fixed with formalin, lightly stained with Nile blue. (× 1200.)

Fig. 5. Very small coccoid forms with thickened walls, 6 weeks at room temperature, liquefied peptone + gelatin culture. Phase contrast. (× 2160.)

Fig. 6. Short motile rods, 4 days at 20°, in galactose + glutamate medium. Phase contrast. (× 1800.)

Fig. 7. Fragmented individual cells washed off cellophan growth over nutrient agar, 6 days at 20°; branched cell at (*a*) has dense polar body. Electron micrograph. (× 5700.)

Fig. 8. Enlargement of Fig. 7 (*a*). (× 17,100.)

Fig. 9. Filament containing four cells, and another spirally twisted one below containing eight cells of which only two are in focus; 10 days at 20° in glucose ammonium sulphate liquid medium. Phase contrast. (× 1440.)

PLATE 2

Fig. 10. Extensive chain formation showing dense bodies more or less evenly disposed along the length of the cells, and clear cross walls; 18 hr. at 30°, on collodion over cellophan over heart broth-casein digest agar. Electron micrograph. (× 8840.)

Fig. 11. Another portion of the specimen in Fig. 10, showing at top thread-like elongation of cell wall after separation of cytoplasmic contents, a pyriform swollen cell in the centre at the end of a chain, and beyond it a V-shaped cell. (× 7820.)

Fig. 12. Another field in the same specimen, showing 'slipping' branching at nodal position of V-shaped cell, with parallel bundles of cells about to follow new orientation. (× 9000.)

Fig. 13. Another field in the same specimen, showing marked inequalities of diameter, with rounding up of cytoplasmic contents at growing tips and stretching of the cell wall; large pyriform cell in centre. (× 10,200.)

Fig. 14. Two rods from portion of 'palisade formation', washed off cellophan over nutrient agar, 24 hr. at 20°. Electron micrograph. (× 26,250.)

Fig. 15. Coccoid and very short rod-shaped cells in palisade and chain formation, showing intercellular connexions and occasionally dense polar bodies; washed off cellophan over nutrient agar, 6 days at 20°. Electron micrograph. (× 14,250.)

Fig. 16. Fine thread-like extension connecting two rod-shaped cells; washed off cellophan over nutrient agar, 6 days at 20°. Electron micrograph. (× 13,680.)

Fig. 17. Chains of older coccoid and short cell elements in which dense polar bodies are more clearly visible; washed off cellophan over nutrient agar 12 days at 20°. Electron micrograph. (× 13,680.)

Fig. 18. Two slender cells cohering in Y-formation, each showing polar body; washed off cellophan over nutrient agar, 4 days at 20°. Electron micrograph. (× 19,500.)

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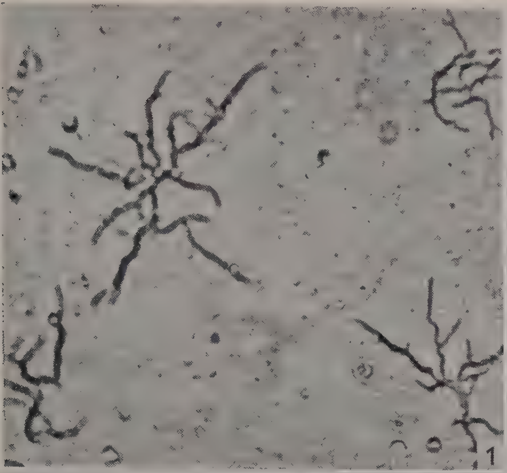
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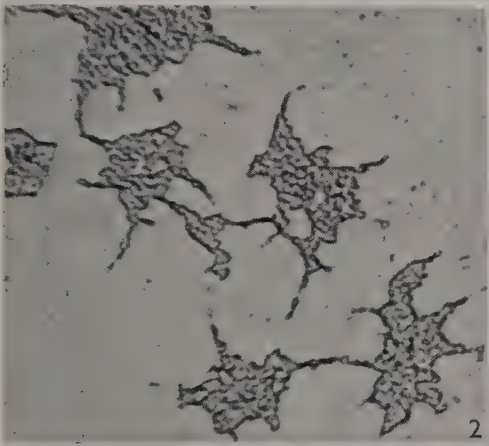
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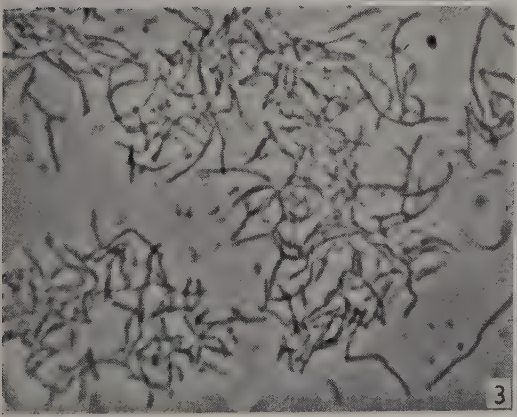
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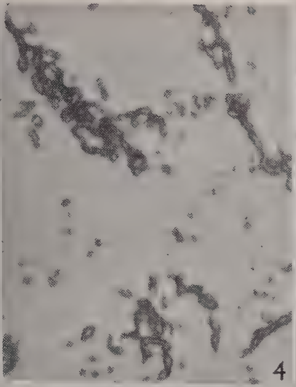
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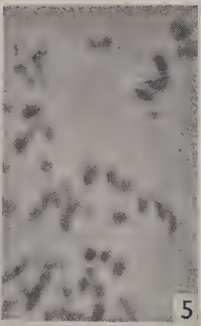
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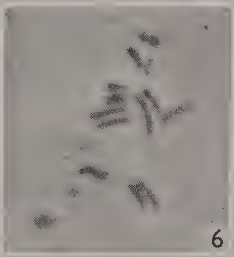
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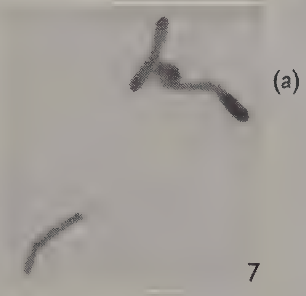

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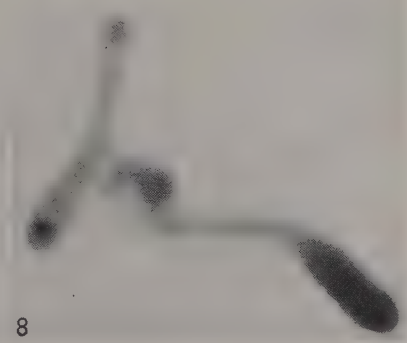
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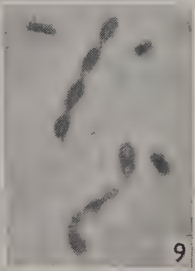
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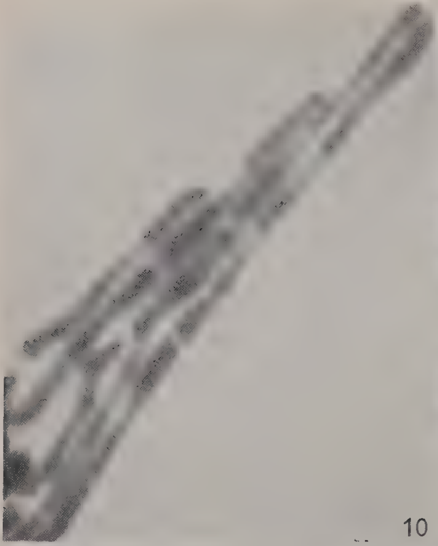
D. ERIKSON—CELL DIVISION IN A 'SOFT' SPECIES OF *NOCARDIA*. PLATE 1



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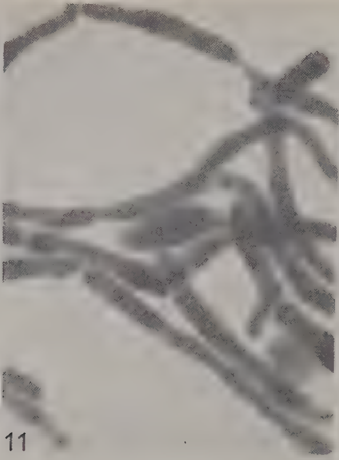

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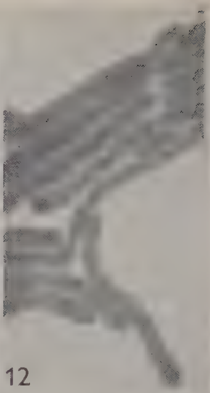

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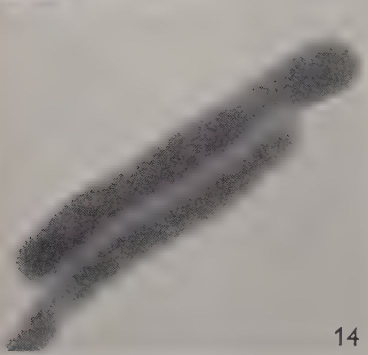

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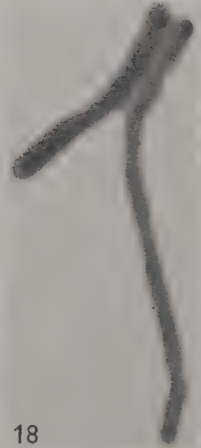

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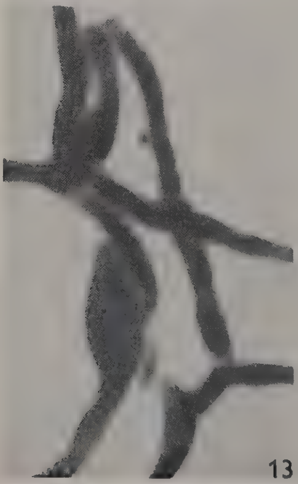

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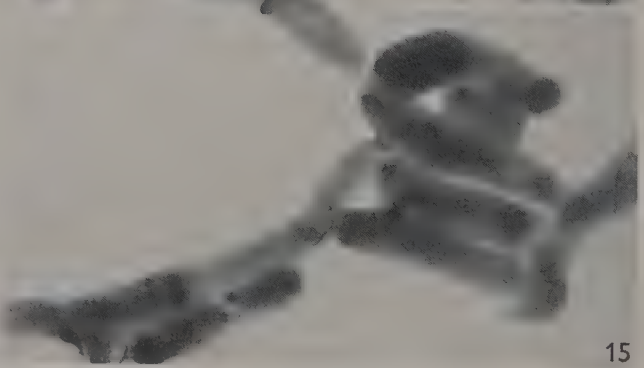

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D. Erikson—Cell division in a 'soft' species of *Nocardia*. Plate 2

ERIKSON, D. & MASSON, F. M. (1954). *J. gen. Microbiol.* **11**, 209–217

Modifications of Micromanipulative Practice Suitable for Single Cell Isolation and Cultivation of (*a*) Aerobic and Transiently Chain-forming, (*b*) Lipophilic and (*c*) Micro-aerophilic Bacteria

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SUMMARY: The methods described allowed successful cultivation of 60 % of single cell isolations in the case of an aerobic, transiently chain-forming and polymorphous organism; 40 % in the case of an organism of related morphology and similar ready viability, but presenting technical difficulties because of the lipophilic nature of the surface membranes; and approximately 2 % in the case of a micro-aerophilic or anaerobic organism of restricted viability.

The commonest test objects for bacterial single cell isolations have been such cells as staphylococci or coliform bacteria which are not only monomorphous in aspect, but which grow and withstand micromanipulative treatment with equal ease. In the course of some years' experience with cells that are constantly polymorphous, and frequently difficult if not impossible to cultivate singly in accordance with the standard technique of de Fonbrune (1949), certain modifications of practice were adopted which form the substance of this communication. Throughout the work a de Fonbrune microforge was used for making the instruments, while for the manipulations the de Fonbrune micromanipulator was employed in conjunction with a Watson Bactil binocular microscope having a 3 mm. apochromatic objective. Illumination was given by a Pointolite lamp. The detailed procedures will be described, under the appropriate group heading, for aerobic and transiently chain-forming organisms, lipophilic organisms and anaerobic and micro-aerophilic organisms.

AEROBIC AND TRANSIENTLY CHAIN-FORMING BACTERIA

Under this heading we have placed those organisms which present no difficulty as regards viability of the individual cell elements, but which produce a coherent complex of cells liable to subdivision in varying ways, while the cells themselves are outstandingly polymorphous. Here the problem was to secure a number of cultures of known provenance (from rod, coccoid cell, filament, etc.), while establishing the mode of growth in each case. *Nocardia turbata* (see Erikson, 1954*a*) was selected as an example of this class.

Methods

Several rows of sterile droplets of medium were deposited in the normal way on the underside of the coverslip of a de Fonbrune oil chamber. In each experiment the position of these droplets was carefully charted on squared paper. The medium employed was nutrient broth. The pool of cells used as inoculum generally consisted of young cells, diluted in sterile medium, taken from a 1- to 2-day old nutrient agar slope. When the desired number of cells had been transferred into the several droplets, their position and shape (long or short filament, with one or more branches, rod or coccoid cell) were noted on the chart. A proportion of separated cells were left to grow in the droplets in the oil chamber under visual inspection. The remaining cells were then transferred one by one to small Durham tubes containing 0·1 ml. medium. Incubation was at 25°. The instruments used for the transference of cells were microloops and micropipettes of varying diameters. Frequently it was found desirable to remove small aggregates of cells from the pool by means of a coarse micropipette to a number of medium-sized droplets, and allow them to grow for 1–2 days before picking out the single cells into small droplets with a small microloop. This ensured vigour of the minute stock of cells dealt with in any one experiment.

In the second stage of the operations—growth from micro-colony to normal culture—those single cells that had multiplied within their droplets in the oil chamber were removed by means of hand-drawn mouth pipettes to Durham tubes containing 0·5 ml. medium; and then, as soon as visible growth appeared, they were transferred by Pasteur pipette to 4 in. culture tubes containing 2 ml. medium. Where the development of separate cells had taken place on agar blocks on slides maintained in a moist chamber, the ensuing growth was taken up (after microscopical observation) in a drop of broth in a mouth pipette and sown into small tubes of liquid medium, thence to nutrient agar slopes. The progeny of all single cells carried the chart labels of their parent on the micromanipulating slide. Single cell isolations were made twice weekly until the development of each type of cell was established. Varying with the ease with which a particular batch of cells could be manipulated, the number of cells isolated was generally between fifteen and thirty in any one experiment. The usual precautions of bacteriological asepsis were observed throughout.

Results

Little difficulty was experienced in working with the cells of *Nocardia turbata* provided they were young, filamentous and non-motile. The very small coccoid cells that have been described in the earlier paper (Erikson, 1954*a*) as resulting from fragmentation of aged growths on nutrient agar or other substrates were subject to Brownian motion and too difficult to see and apprehend under the 3 mm. dry objective used throughout the micromanipulating operations. The short motile rods and filaments could be picked up from time to time, but their pursuit was necessarily slow. It was found easier and quicker to remove non-motile filaments and rods for experimental work, while noting the position and observing the subsequent growth of any coccoid or motile cells left behind in the droplets. All cells developed in the same way—by elongation, branching, subdivision into small mycelia—but the rate of progress varied with different cells, and subdivision might frequently precede branching.

The following is an example of vigorous unchecked growth. The medium employed for the droplets was nutrient broth, the temperature at which the manipulating slide was incubated (in a moistened metal slide box with tight fitting lid) between operations was 25°. In one droplet (A) four short filaments were deposited from a pool of 1-day cells taken from a nutrient agar slope.

1st day: each filament increased in length;

2nd day: filaments had grown together into a small mycelium already showing fragmentation, with a few motile cells at large; nine long filamentous cells were transferred to separate droplets;

3rd day: five of the nine filamentous cells had grown with 6–8 branches; many of these cells were already fragmented, or separated apart with the intrusion of a microloop; in some instances small coccoid cells were beginning to develop. The progeny of the five single cells were now removed by microloop to separate Durham tubes containing 0·5 ml. medium as follows:

A1 5 filaments transferred: 1 long filament and 1 short coccoid cell remain in droplet;
A2 6 long cells transferred and 1 very short cell remains;
A3 5–6 long filaments transferred; no cells remain;
A4 2 filaments removed and 2 short cells remain;
A5 (1) 3 out of 7 filaments in drop removed;
A5 (2) another 3 filaments removed; 1 left in drop.

Meanwhile those portions of the original filaments left in droplet A, which had been replenished with fresh medium by means of a micropipette, have grown again into a ramifying network showing fragmentation with the production of some actively motile cells; of these 13 were removed to separate sterile droplets;

4th day: 9 of the 13 cells had multiplied, and the microcolonies were removed to little tubes—series B; when replenished with medium the coccoid cells remaining in A1 and A2 had elongated; 6 other single filaments from another sub-pool transferred directly by means of a micropipette carrying minute agar columns (de Fonbrune, p. 165) to Durham tubes—series G;

5th day: A2, A3, A5 (1), in the little tube series all showed visible minute granular growth with clarity of medium; the short cells that had elongated in droplets A1 and A2 were now showing fragmentation of their branched filaments;

6th day: 7 of series B and 3 of series G now showed tiny granules of growth with clarity of medium in the small tubes; in the 3 tubes of the A series the granular growth had now disintegrated and multiplied with the result that there was a slight turbidity of the medium;

7th day: the granular growths in series B and G were disrupted with a diffusion of cells through the slightly turbid medium; normal mass cultures were obtained from all these tubes.

Sixty per cent of all attempted single cell isolations of *Nocardia turbata* were successful, and it required 7–10 days for the preparation of a macroculture from a single cell. The repeated separation into fresh droplets of the different types of cells as they appeared allowed visual inspection of every stage of growth, from an individual cell point of view. On the other hand, the sowing of such single cells of varying shapes into small tubes of media made evident a stage which could not be demonstrated by other means. Because of surface tension factors and the varying depths of the droplets, single cells are frequently apt to divide into a number of rods or coccoid cells (see history of droplets A1 to A5), before branching or chain-formation take place. In the very much larger volume of liquid contained in a small tube (0·5 ml.), this early subdivision apparently did not take place, and the organism was able to produce the initial coherent growth characteristic of its family group. In this way only was it possible to demonstrate a phylogenetic relationship, since the use of even very dilute suspensions as inocula for mass cultures constantly resulted in turbidity of growth from the beginning.

LIPOPHILIC BACTERIA

This class comprises organisms which grow readily on suitable substrates, but which offer considerable resistance to the manipulator because of the nature of their surface membranes. The example taken was a paraffin-utilizing organism, *Nocardia sebivorans* (see Erikson, 1954*b*). This species is further distinguished from *N. turbata* by the relative stability of the cell-mass formed (liquid cultures being consistently clear), and by the development of a secondary aerial growth the component cells of which are hydrophobe in nature.

Methods

When attempts were made to grow *Nocardia sebivorans* in the same manner and in the same complex nutrient broth and agar as had been used for *N. turbata*, which it was thought would afford the maximum chances of survival for isolated cells, various difficulties of a practical nature were encountered at the outset. They were concerned with the following points.

(1) *Nature of the inoculum.* A 1-day growth on nutrient agar required mechanical disruption by shaking, which was readily accomplished but with a consequent diminution of viability. A 7-day growth in similar circumstances yielded a non-homogeneous suspension of fragments from the surface network of filaments together with short aerial cells. These very short cells proved difficult to manipulate in numbers, although several individuals were isolated successfully. Similar objections applied to the material taken from the surface growth on liquids, while the bottom growth in nutrient broth appeared to have a surface coating that resisted seizure by the micro-instruments. The most suitable material was obtained by growing the organism in a simple Czapek glucose nitrate liquid medium, subculturing daily 3 or 4 times, finally taking a small portion of the bottom growth for the initial pool. The longer filaments so obtained separated readily in the droplets, and, since they appeared more refractile than the majority of the cells tried previously, could be seen and picked up more quickly.

(2) *Effect of prolonged growth at 37° upon droplets.* Even with the precaution of placing a large drop of water in the well of the oil chamber, a number of small droplets of medium dried up during the necessary 2–3 days' incubation at 37°, and frequent replenishment by micropipette was needed. A more serious matter, when nutrient broth was used for the droplets, was the occasional formation of a thin skin at the oil/medium interface after incubation. The skin was quite tough, resisting the passage of micro-instruments, and no growth at all took place of any cells within such droplets. It appeared, therefore, that the film prevented the diffusion of air into the droplets. The same broth supported good growth of the organism in ordinary tube cultures, and it may be that the batches of broth which yielded such results were slightly richer in fatty substances than usual, and with incubation this concentration was enhanced. It is known that solutions of sterols and other fatty substances as well as of proteins have the property of forming monolayers, not only at air/water interfaces, but also at oil/water interfaces (see Sexton, 1953, p. 70). When the broth was diluted, the phenomenon did not occur. In general, it was found best to employ Czapek glucose nitrate medium for the droplets.

(3) *Attraction of oil phase for the growing cells.* The lipophilic nature of the organism derived from non-fatty media was very evident when small aggregates of cells were placed in sub-pools, and within a matter of seconds had swung over to the boundary of the drops, generally with the longer branching filaments projecting into the paraffin oil of the chamber. When single cells were placed in droplets, they tended to lie close and with their long axes parallel to the boundary. After incubation, it seemed merely a matter of chance whether or not the resultant growth was still partially contained within the droplet. In fact, it was frequently found that when a small complex of cells had developed at the margins of a droplet, it migrated overnight into the oil phase and was lost. While, on the other hand, droplets which had been sterile, and to which nothing had been consciously added, might become the hosts of one or more cells or aggregates of cells that were at large in the oil phase. There was, of course, no question of motility on the part of any of these cells, merely one of physical attraction (cf. Webley, 1953).

Different oils were then tried as substitutes for paraffin oil. The vegetable oils such as olive oil, castor oil, etc., were unsatisfactory, either because of poorer visibility or because they did not permit good growth. Polyric oil (Edward Gurr Ltd.), which is used as an immersion oil, allowed very good growth and visibility, but it was found that the aqueous droplets did not adhere very well to the coverslip, and the growing cells were readily attracted into the oil phase. Non-oily substances that are non-miscible with water such as diethyl phthalate were not sufficiently viscous for incubation in the chamber. The use of the standard paraffin oil chamber was therefore continued, but as a rule only for the minimum period of time required for separating and charting the cells. Many pools of cells might be grown successfully and their course of development observed in the manipulating slide, but because of the lack of effective boundary at the oil/medium interface, individual cells were removed by micropipette to small tubes or slide cultures.

Cultivation of the small Durham tubes of liquid media was as previously described. Slide cultures employing solid media were made in order to obtain visual inspection of the development of the separate cells. The latter were delivered on to the moistened surface of a small block of nutrient agar on a slide in a sterile Petri dish, the block being immediately capped with a sterile coverslip and the cover of the dish replaced. When 18–20 slides were prepared, they were lifted with sterile forceps on to metal trays, each of which slid horizontally into its place in the internal scaffolding of a sterile metal container (see Pl. 1, Figs. 1, 2). The upper and lower sectors contained sterile wadding which was moistened with water, and so maintained a very efficient moist chamber during prolonged incubation (1–2 weeks). The container used was an especially stout cupro-nickel drum built to withstand evacuation for anaerobic cultivation, the only gas ingress being at the two leads which were fitted with pieces of cottonwool-plugged sterile glass tubing. It is probable that a less substantial tin or glass container would serve equally well for aerobic growth. The use of a single container for a number of slides was, however, found preferable to a large number of individual Petri dishes lined with moistened filter-paper. Where it was desired to follow the development of the secondary aerial cells, without the restriction imposed by coverslips, individual slide boxes were employed. These were made of the same cupro-nickel as the large vessel, had close-fitting lids, and were provided with two ledges on which the slide rested, while the space below was packed with sterile moistened absorbent cottonwool (see Fig. 1). Such slide boxes, made in two widths to fit ordinary slides or the broader micromanipulating slide, had the advantages of maintaining the slide cultures in position, preserving humidity over considerable periods, and economizing in incubator space.

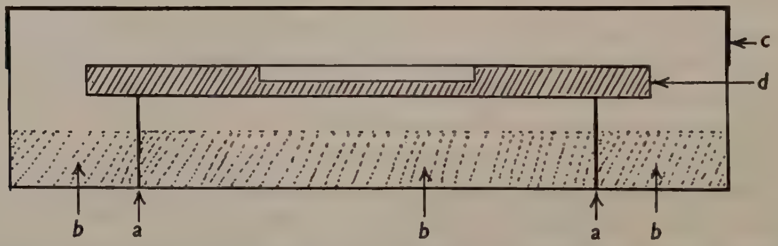


Fig. 1. Slide box for moist incubation of droplets containing single cells. ×1. *a*, *a*, supports extending whole width of box; *b*, moistened absorbent cottonwool; *c*, tight-fitting lid. *d*, standard manipulator slide with coverslip and oil chamber.

Results

Forty per cent of all attempted single cell isolations of *Nocardia sebivorans* were successful, and it required 3–4 weeks for the preparation of a mass culture from a single vegetative cell. This lengthy period was due in part to the necessity of the repeated transfers that have been described, and to the several lags so induced. Where growth could be kept under observation in the droplets on the slide, and the microcolony then removed to a small culture tube in the normal way, cultivation was more rapid. Similarly, in those instances where the very minute aerial cells of a culture were transferred by microloop directly to small Durham tubes of nutrient broth, tiny granules of visible growth appeared within 2 days, and the mass culture could be obtained in 7–10 days. On the whole, more consistent results were secured with the slower methods.

ANAEROBIC AND MICRO-AEROPHILIC BACTERIA

In his account of the paraffin oil chamber as a menstruum for the cultivation of aerobic micro-organisms within aqueous nutrient droplets, de Fonbrune (1949) demonstrated that sufficient air would diffuse through the oil to enable the strictest aerobes to grow. He further stated (p. 136) that 'Par contre, nous n'avons pu obtenir, dans ce dispositif, des cultures de germes anaérobies, même en purgeant préalablement l'huile des gaz qu'elle tenait en dissolution.' We can confirm this, having tried very many times to cultivate the anaerobic *Actinomyces israelii*, which is sometimes regarded as micro-aerophilic, within the oil chamber, and having constantly failed in spite of the following precautions: flushing with nitrogen the tube containing the parent inoculum as well as the micromanipulating slide and the medium used for the sterile droplets just before operations, and removing the finished slide to the anaerobic jar immediately afterwards. No growth whatever occurred, no matter what additions were made to the medium, although minute just visible granules of cell material transferred to small Durham tubes containing suitable liquid substrate gave rise to good growth when incubated in an anaerobic jar. Accordingly, it seemed advisable to have recourse to the principle adopted with the lipophilic organisms (see previous section), and not to allow the cells to remain in the manipulating slide any longer than necessary.

Methods

The characteristic granular growth of *Actinomyces israelii*, which always leaves the liquid substrate clear, required mechanical disruption for the purposes of obtaining an initial pool of clearly visible cells; the cells which tended to fragment most easily were the shortest cells, which were correspondingly difficult to manipulate. In this group of organisms the cells which are compelled to grow against a plane surface, whether nutritive like agar or non-nutrient such as cellophan or coverslip, have frequently been found (Erikson, 1949) to be proportionately longer than those which have been allowed to develop unimpeded in a liquid substrate. Consequently, the most suitable cell material from a visual and handling point of view was the 5 to 7-day growth between coverslip and agar block that had developed in slide cultures incubated in the anaerobic drum. The small diphtheroid forms more common in the break-up of liquid growth were also employed. The type of each cell unit was charted as usual. The medium employed throughout was the stock (S) medium (glucose + heart broth + casein digest) of Erikson & Porteous (1953).

Individual cells were transferred by micropipette to:

(i) small Durham tubes containing liquid broth;

(ii) similar tubes containing the same medium with the addition of 0·2 % agar, melted and held at 45°;

(iii) the same medium containing 0·1 % agar and which filled approximately 1 in. of glass tubing (*c.* 4 mm. diam.) held diagonally within a small screw-capped bottle by a layer of solid (2 %) agar medium at the bottom (see Fig. 2); after inoculation, during which operation the micropipette was plunged into the viscous medium inside the tubing and the contents washed out by repeated sucking-up and explusion, the bottle was cautiously filled up to the lip and over the top of the tubing with sterile boiled water from which all air had been driven; and the metal cap screwed tightly down;

(iv) the moistened surface of agar (2 %) blocks of medium on slide cultures, which were then placed in the anaerobic drum described in section (*b*), after being capped with sterile coverslips.

Cultures (i) and (ii) were incubated in an anaerobic jar in the ordinary way; and the anaerobic drum (iv) was also evacuated and filled with nitrogen or hydrogen; cultures (iii) were incubated directly. The temperature of incubation throughout was 37°, and the time of incubation 7–14 days.

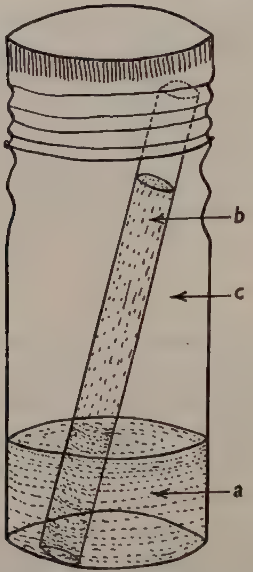
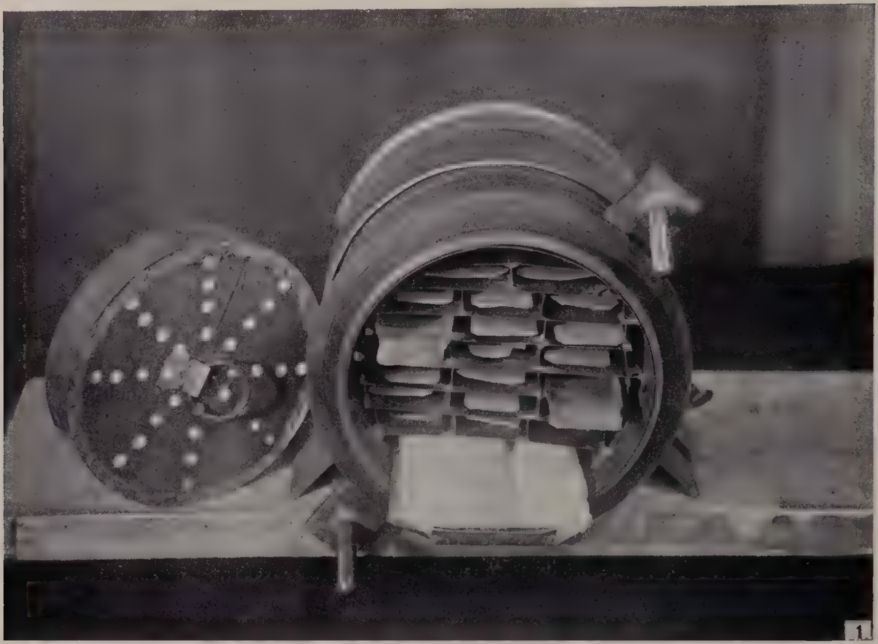
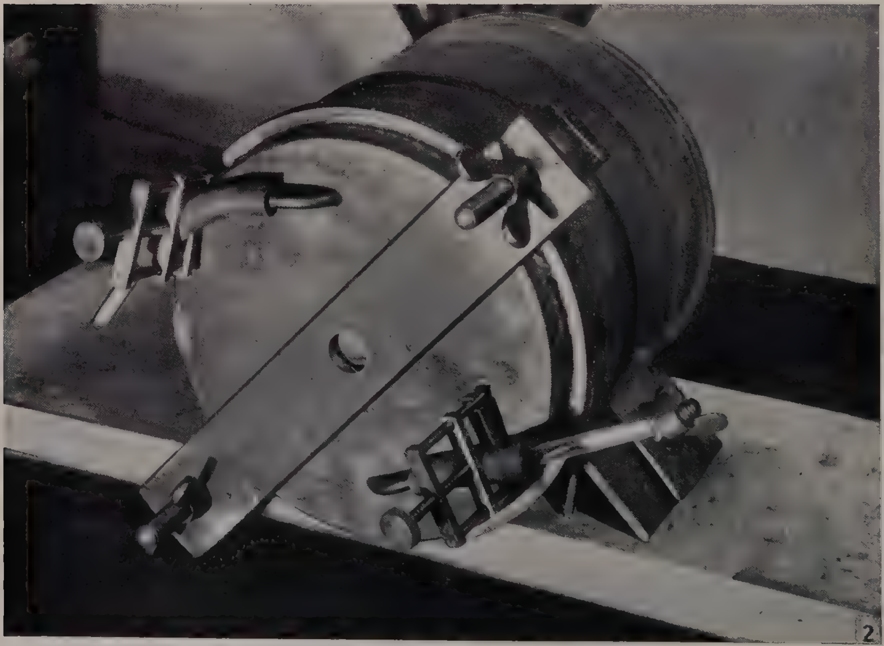


Fig. 2. Screw cap bottle for anaerobic cultivation from a single cell. × 1·5. *a*, 2 % agar medium; *b*, 0·1 % agar medium; *c*, sterile, freshly boiled water.

Results

Only 2·3 % of attempted single cell isolations of *Actinomyces israelii* were successful, and it required 2–3 weeks for the preparation of a mass culture from a single cell unit. The distribution of successes according to methods was as follows: in (i) none out of 37 attempted; (ii) 2 out of 43; (iii) 1 out of 33; (iv) 1 out of 60. The superiority of the semisolid to the liquid or solid medium





D. Erikson & F. M. Masson—Modified methods of single cell cultivation.
Plate 1

is evident. It is also noteworthy that relatively more profuse growth occurred more quickly in (iii), where the semisolid medium was contained in a continuous aqueous phase than in (ii), which was incubated in a gas phase. The necessity of making large numbers of slide cultures to watch the growth-stages of cells whose viability ratio is so low is also apparent. Yet, on the whole, by means of the combination of methods described here the proportion of successes was as high or higher than would be expected of an anaerobic organism well known as not easy in cultivation.

The senior author (D. E.), a member of the scientific staff of the Agricultural Research Council, wishes to thank Prof. J. Cruickshank for the hospitality of his department; and the Director and Dr A. E. Oxford of the Rowett Research Institute for permission to use their micromanipulator and microforge continuously for long periods. We also wish to thank Dr J. W. Porteous of Aberdeen University and Mr G. Masson of the Rowett Research Institute for help in designing and constructing the anaerobic slide-carrier drum; and Messrs Accles and Pollock Ltd., Oldbury, Birmingham, for a gift of fine cupro-nickel tubing for use in micromanipulation.

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EXPLANATION OF PLATE

Fig. 1. Anaerobic drum, with outer lid removed, showing inner lid, lined with gauze at side, and internal scaffolding carrying slide trays. One large tray with moistened wadding is pulled out of position at bottom. Another similar tray fits into the top compartment.

Fig. 2. Anaerobic slide-carrier drum, battened down and fastened to board, with lid containing the gas leads tightly screwed in place.

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Dissimilation of Citric Acid by *Aerobacter aerogenes* and *Escherichia coli*

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SUMMARY: When *Aerobacter aerogenes* was harvested from citrate + mineral salt media in which the concentration of carbon source limited the crop, citridesmolase activity of cells was high but was absent when growth ceased with citrate in excess. These differences were abolished by disintegration of the cells. The ability of *Escherichia coli* to dissimilate citrate was measurable only after growth in a medium containing peptone, citrate and glucose: when the latter was consumed activity fell and was revived by further glucose additions. Differences in activity of such cells were not abolished by their disintegration. Cells lost their activity when incubated for 30 min. with phosphate buffer or with the citrate + mineral salt medium that supports growth of *A. aerogenes*. *E. coli* grown at the expense of glucose in a mineral salt medium + citrate, did not utilize citrate for growth during the first day and no citridesmolase activity could be detected, but when subsequently tested in a medium which permitted development of activity, higher activities were reached by cells that had grown in presence of citrate. *E. coli* grown in peptone + citrate + glucose medium possessed little oxaloacetate decarboxylase; by the action of disintegrated cells, citrate gave rise to equimolar quantities of keto-acid, chiefly oxaloacetic acid.

A citrate + mineral salt medium will support growth of most strains of *Aerobacter aerogenes* but not of *Escherichia coli* (Koser, 1924). The extraction of 'condensing enzyme' from *E. coli* (Stern & Ochoa, 1951) and the demonstration (Lara & Stokes, 1952) that dried cells oxidized citric acid indicated a function for this compound in the economy of *E. coli* and suggested that inability to metabolize citrate might be attributed to its failure to pass into the cells. From studies of conditions affecting the ability of *A. aerogenes* to dissimilate citrate, and from experiments with cell-free extracts, Dagley & Dawes (1953*a*, *b*) concluded that dissimilation to oxaloacetate and acetate (Brewer & Werkman, 1939) occurred by an inducible (adaptive) mechanism that was not identical with reversal of citrate synthesis in the tricarboxylic acid cycle. Gillespie & Gunsalus (1953), working with *Streptococcus faecalis*, arrived at similar conclusions and gave the name 'citridesmolase' to the enzyme responsible for citrate cleavage. These results suggest that although difficulties of cell permeability may be sufficient to account for the inability of *E. coli* to oxidize citrate, failure to dissimilate this compound may be due also to lack of citridesmolase development in cells grown in citrate + mineral salt media. Lominski, Conway, Harper & Rennie (1947) and Vaughn, Osborne, Wedding, Tabachnick, Beisel & Braxton (1950) reported disappearance of citrate from cultures of *E. coli* supplied with various nutrients, while Grunberg-Manago & Gunsalus (1953) prepared extracts of the organism which dissimilated citrate. In the present work the nutritional requirements for citridesmolase development in *E. coli* have been studied and compared with those for *A. aerogenes*. Further, an attempt has been made to evaluate the contribution of cell permeability to whole-cell activity by conducting experiments with suspensions of disintegrated cells.

METHODS

The organisms used were *Aerobacter aerogenes* NCTC 418 and *Escherichia coli* NCTC 5928. In a mineral salt medium containing citrate as sole source of carbon, the concentration of the citrate may limit the crop of *A. aerogenes*, or citrate may remain unconsumed when growth has ceased (Dagley & Dawes, 1953*a*). Media used contained: KH_2PO_4, 50 g.; $(NH_4)_2SO_4$, 20 g.; $MgSO_4.7H_2O$, 2 g.; made to 10 l. with distilled water, with the addition of either 70 g. or 200 g. sodium citrate dihydrate to provide limiting or excess citrate respectively. The medium for growth of *E. coli* contained: KH_2PO_4, 90 g.; peptone (Difco), 56 g.; $MgSO_4.7H_2O$, 2 g.; sodium citrate dihydrate, 50 g.; glucose, 10 g.; water to 10 l. All media were adjusted to pH 7·0 by addition of 5N-NaOH. Batches of both organisms were grown at 37° in 10 l. flasks filled to the neck and were harvested in a Sharples supercentrifuge. Suspensions of disintegrated cells were prepared by crushing *c.* 10 g. frozen cell paste in a Hughes (1951) press previously cooled to −14°. Crushed cells taken up in 40 ml. phosphate buffer (pH 7·0) gave rather viscous suspensions which, when active cells of both species were used, after centrifugation at 7000 ***g*** to remove debris provided translucent cell-free extracts capable of dissimilating citrate. The abilities of cells to dissimilate citrate (citridesmolase activity) may be compared by measurement of initial rates of keto-acid production by suspensions of standard turbidity (Dagley & Dawes, 1953*a*). This procedure was adopted in the present work, with slight modifications due to the fact that *E. coli* was not in general so active as *A. aerogenes*. Samples were taken from cultures and the bacteria removed by centrifugation: they were well drained but not washed before testing since activity may be considerably decreased by washing. The volume of any sample was such that the final concentration of cells was 2×10^9/ml. when the bacteria were resuspended in test medium (sodium citrate dihydrate 2·1%; KH_2PO_4, 0·5%; $MgSO_4.7H_2O$, 0·04%; pH 7·0). After incubation for 15 min. at 37° total keto-acid concentration in the supernatant after centrifugation was determined by the method of Friedemann & Haugen (1943). Pyruvate and oxaloacetate were extracted as their 2:4-dinitrophenylhydrazones (Dagley, Fewster & Happold, 1952). The R_F values of the oxaloacetate and α-ketoglutarate derivatives in the butanol/ethanol/ammonium carbonate buffer mixture used by these authors are similar, 0·25 and 0·20 respectively; the glycine/NaOH solvent of Virtanen, Miettinen & Kunttu (1953), which separates them more readily, was also used. Chromatography of 2:4-dinitrophenylhydrazones is complicated by the existence of stereoisomers (Isherwood & Cruickshank, 1954), although 'satellite' spots were detected in the above solvents only in the case of pyruvate; accordingly, no experiments are described in which identification rests solely on R_F values.

Glucose concentrations were determined by the Somogyi (1937) method; total nitrogen in 3 ml. samples of suspensions of disintegrated cells by a modification of the Kjeldahl procedure (Varley, 1954); growth was followed turbidimetrically as described by Dagley, Dawes & Morrison (1950). Conventional Warburg techniques were used as described by Umbreit, Burris & Stauffer (1945).

RESULTS

Citridesmolase activity of Aerobacter aerogenes: *comparison of whole and distintegrated cells*

Dagley & Dawes (1953*a*) showed that cells were active when they were harvested during the period of logarithmic growth in a citrate mineral salt medium, or when growth had ceased through citrate depletion. Cells from a culture in its stationary phase with excess citrate were inactive. This loss of ability to dissimilate citrate was now studied. A suspension of disintegrated cells was prepared from an active batch (0·8 μmole keto-acid/2×10^9 cells/15 min.) and its nitrogen content determined. Dilutions (1/5, 1/10 and 1/100) were made in phosphate buffer (pH 7·0), 2·5 ml. of each dilution added to separate volumes of citrate test medium in 6×1 in. Pyrex tubes and after shaking to disperse the suspensions they were incubated for 15 min. at 37°. Equal volumes of 10 % (w/v) ice-cold trichloroacetic acid were then added, the precipitates filtered off and the keto-acid concentrations of the filtrates determined. The experiment was repeated with cells which had no detectable activity harvested from medium containing excess citrate. In Fig. 1 it is seen that keto-acid production was proportional to the amount of suspension. This relationship ceased at high values, an increase from 164 to 820 μg. N/ml. (415 %) only gave a 24 % increase in keto-acid production. In Fig. 1 are also shown results for disintegrated cells grown with aeration in a glucose + mineral salt medium; such preparations when tested in the Warburg respirometer were able to oxidize citrate but were unable to dissimilate it appreciably. It is clear from Fig. 1 that the large difference in citridesmolase activity shown by cells from media containing different concentrations of citrate was largely abolished when the cells were disintegrated.

Influence of the growth medium on citridesmolase activity of Escherichia coli

Peptone + citrate + glucose medium was inoculated with a suspension of *Escherichia coli* prepared from an agar slope and growth, citridesmolase activity (whole cells) and glucose consumption were measured at suitable time intervals. Citridesmolase activity increased whilst glucose was consumed and fell when it was exhausted (Fig. 2). It had already been observed for several cultures that activity depended on the time they remained in the incubator after cell division had ceased. At 395 min. after inoculation the culture was transferred to the cold room (4°) for 20 hr. and then returned to 37°. Activity decreased in this period, but on addition of glucose was rapidly regained.

On resuspension in an equal volume of phosphate buffer (pH 7·0) for 30 min. at 37°, cells with high activities produced no keto-acids from the citrate test medium. When Na citrate dihydrate (2·1 %), $(NH_4)_2SO_4$ (0·2 %) and $MgSO_4.7H_2O$ (0·04 %) were added to the buffer, furnishing a medium able to support growth of *Aerobacter aerogenes*, the activity of *E. coli* was lost just as rapidly and no growth occurred. Losses in activity of 70–80 % occurred when *E. coli* cells were washed twice with water. Cells that had lost activity by these treatments regained it completely within 30 min. when incubated with the complete peptone + citrate + glucose medium, but not when any one of these three constituents was omitted.

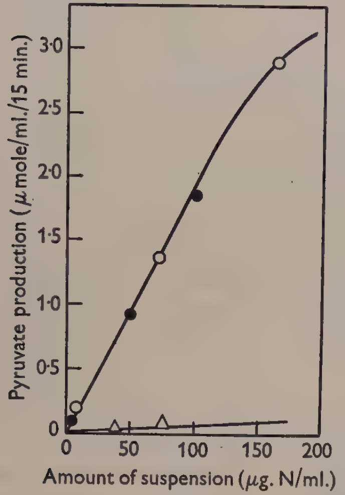

Fig. 1

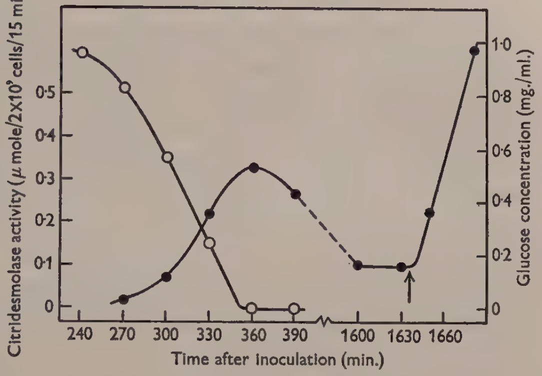

Fig. 2

Fig. 1. Relations between pyruvate production from citrate and amounts of disintegrated cell suspension of *Aerobacter aerogenes* in the incubation mixture. Citrate exhausted in growth, whole cells active, ●; citrate in excess, whole cells inactive, ○; growth in aerated glucose-mineral salt medium, whole cells inactive, △.

Fig. 2. Development of citridesmolase activity of *Escherichia coli*: dependence on presence of glucose in growth medium. Activity, ●; glucose concentration, ○; further glucose addition at point indicated by arrow. At 395 min. the temperature of the culture was reduced to 4° and at 1600 min. incubation was resumed at 37°.

Suspensions of disintegrated cells were prepared from a series of batches with different activities resulting from various incubation periods in the stationary phase. Relationships were established, as described for *Aerobacter aerogenes* between nitrogen content of diluted preparations and their ability to produce keto-acids from citrate (Fig. 3). The results were in sharp contrast to those obtained with *A. aerogenes* since low activity was not increased by disintegration: there was a rough proportionality between activity before and after crushing. Furthermore, when one-half of a large batch of active cells of *Escherichia coli* was incubated with an equal volume of buffer for 30 min. both whole cells and those subsequently disintegrated were devoid of activity. The other half of the batch, crushed immediately after harvesting, was active after disintegration and an active cell-free extract was obtained from the preparation by centrifugation. The medium used in tests for activity contained Mg^{++} ions which Dagley & Dawes (1953*b*), showed to be a co-factor for citridesmolase activity in *A. aerogenes*. Gillespie & Gunsalus (1953) showed activation of the enzyme from *Streptococcus faecalis* by Mn^{++} ions, but concentrations up to 10^{-3}M did not restore citridesmolase activity to whole or disintegrated *E. coli* cells after it had been lost by treatment with buffer.

Effect of growth in glucose + citrate + mineral salt medium on subsequent development of citridesmolase activity in Escherichia coli

Vaughn *et al.* (1950) concentrated on ability of *Escherichia coli* to decompose rather than grow on citrate since observations of growth in media containing a variety of compounds showed that 'the added compounds functioned only to provide food for the growth of the test cultures'. Measurements of citrate utilized were made after 4 days. Experiments were now conducted to ascertain whether additional growth could be observed in glucose + mineral salt media after prolonged incubation in the presence of citrate. One series of 6 × 1 in. Pyrex boiling tubes each contained 25 ml. of a solution (sol. A) of: KH_2PO_4 (0·72 %, w/v, pH 7); $(NH_4)_2SO_4$ (0·12 %, w/v); $MgSO_4.7H_2O$ (0·4 %, w/v); glucose to give a concentration range 0–9 mM.; similar series contained in addition Na citrate dihydrate (0·44 %, w/v). Determinations of growth of *E. coli* (Table 1) at 24 hr. showed no significant difference between the tubes that contained citrate and those that did not, and analysis at that time showed that glucose had disappeared in all cases. On prolonged incubation, however, further slow growth occurred, which ceased after 64 hr., in cultures containing citrate. Citridesmolase was not detected in these cultures at any stage, but the presence of citrate was not without effect even during the first 24 hr. when growth occurred entirely at the expense of glucose. This is shown by the following experiment. Glucose (0·08 %, w/v; 4·4 mM.) was added to two 250 ml. lots of sol. A; to one lot no further addition was made and to the other 0·44 %, w/v, Na citrate dihydrate was added. After 18 hr. growth the cells were centrifuged and resuspended in an equal volume of the peptone + citrate growth medium for *E. coli*, from which glucose had been omitted. Cells re-

Table 1. *Relation between crop of* Escherichia coli *and glucose concentration in mineral salt medium, with and without citrate additions*

Glucose concentration (mM)	No of bacteria (millions/ml.) at times indicated			
	Citrate absent		Citrate present	
	24 hr.	68 hr.	24 hr.	68 hr.
0·89	90	90	90	110
1·78	180	195	195	360
2·67	250	280	275	740
3·56	320	340	315	585
4·44	395	390	390	700
8·89	620	790	750	960

moved from either batch 30 min. after resuspension showed no activity, but 15 min. after adding 1 g. glucose/l. to each an increase in activity was detected for those cells which had grown in the presence of citrate (Fig. 4). For those grown without citrate there was a lag period and the maximum activity attained was less. This was confirmed in experiments using different glucose concentrations in the peptone + citrate + glucose medium and the results are given in Table 2. Clearly growth in mineral salt medium in the presence of citrate, although not conferring measurable citridesmolase activity, enabled a given amount of glucose in peptone medium to produce subsequently a greater activity in the cells.

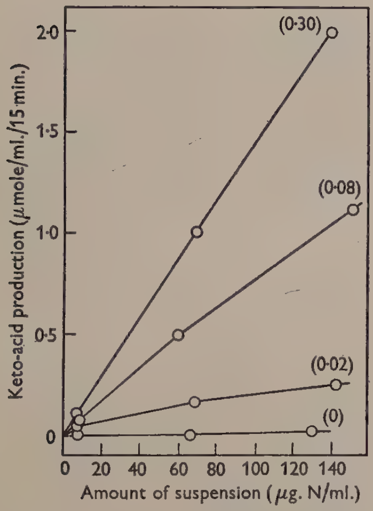

Fig. 3

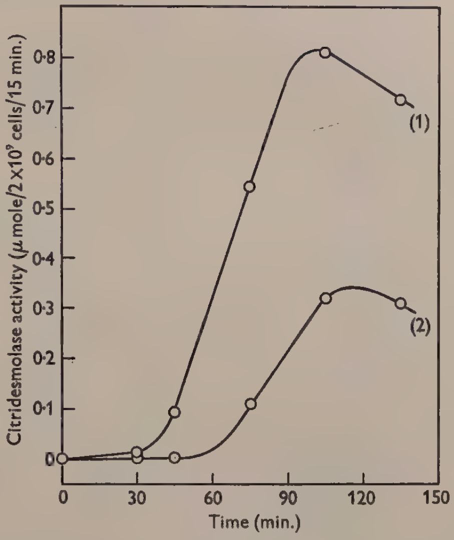

Fig. 4

Fig. 3. Relations between keto-acid production from citrate and amounts of disintegrated cell suspension of *Escherichia coli* in the incubation mixture. Figures in brackets give activities (μmole keto-acid/2 × 10^9 cells/15 min.) of whole cells before crushing.

Fig. 4. Effect of addition of citrate to a glucose + mineral salt growth medium on citridesmolase development in *Escherichia coli*. After growth, cells were suspended in peptone-citrate medium to which glucose was added at 30 min. and development of activity followed with time. Citrate present during growth in glucose + mineral salt medium, (1); no citrate present, (2).

Table 2. *Dependence of citridesmolase development on glucose concentration*

Escherichia coli grown in glucose + mineral salt medium, with or without addition of citrate, and transferred to peptone + citrate media containing various concentrations of glucose. The maximum activity (see Fig. 4) was then measured in each case.

Glucose concentration (g./l.)	Maximum activity (μmole/2 × 10^9 cells/15 min.)	
	Citrate present	Citrate absent
0·0	0·00	0·00
0·2	0·05	0·00
0·4	0·25	0·02
0·6	0·53	0·07
0·8	0·83	0·30

Products of citridesmolase action

Cell-free extracts of *Aerobacter aerogenes* dissimilate 1 mole of citrate to 1 mole each of pyruvate, CO_2 and acetate; 1 mole of oxaloacetate is formed initially but the preparations contain a powerful oxaloacetate decarboxylase (Dagley & Dawes, 1953*b*). To each of a series of tubes containing 2·5 ml. of phosphate buffer (KH_2PO_4, 0·5 %; pH 7) and 0–3·5 μmole citrate/ml. was added 0·5 ml. of an active disintegrated suspension of *Esherichia coli.* After 10 min. incubation total keto-acid concentrations were determined and the values plotted against added citrate (Fig. 5). The slope of the linear part of the curve gives a citrate: keto-acid ratio of 1·06. Extraction and chromatography of the 2:4-dinitrophenylhydrazones using the solvents of Dagley *et al.* (1952) and of Virtanen *et al.* (1953) gave well-defined spots corresponding to oxaloacetate (R_F values 0·25 and 0·8 in the two solvents, respectively) in addition to weaker spots corresponding to pyruvate. When fluids were deproteinized after action of the disintegrated cells and held at 100° for 15 min. before 2:4-dinitrophenylhydrazone formation, the spot of R_F value 0·25 disappeared and gave place to the two spots for pyruvate of which the slower-moving was most distinct. Similar fluids treated with aniline citrate in a Warburg respirometer evolved *c.* 0·8 μmole CO_2/μmole keto-acid estimated as 2:4-dinitrophenylhydrazone. Suspensions of disintegrated *E. coli* showed very little oxaloacetate decarboxylase activity.

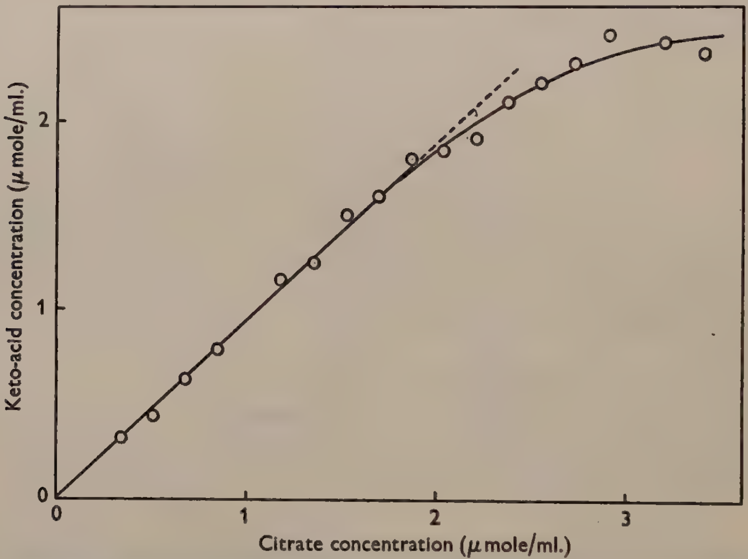

Fig. 5. Production of keto-acids from various concentrations of citrate by a suspension of disintegrated *Escherichia coli.*

DISCUSSION

The results for *Aerobacter aerogenes* present certain marked contrasts to those for *Escherichia coli.* Glucose inhibited citridesmolase development in the former when growing in a citrate + mineral salt medium (Dagley & Dawes, 1953*a*), whereas activity of *E. coli* increased during glucose consumption and decreased when glucose had disappeared. Further, this loss of activity persisted when the cells were disintegrated while differences in activity for *A. aerogenes* grown in citrate media without aeration were abolished by crushing. Accumulation of acetate in growing cultures of *A. aerogenes* has been associated with the concomitant loss of citridesmolase activity (Dagley & Daws, 1953*a*); acetate may inhibit transport of citrate to its site of action within the cell. Relevant to this suggestion are the conclusions of Barrett, Larson & Kallio (1953) that a carrier system is involved in citrate transport, and those of Kogut & Podoski (1953) that such a system may be developed adaptively (by induction).

The labile nature of the citridesmolase of *Escherichia coli* and its rapid reactivation only when complete peptone + citrate + glucose medium is used may be related to one view of enzyme synthesis discussed by Spiegelman & Halvorson (1953). Their results suggest that if an enzyme precursor is formed it may become active enzyme only with participation of the free amino acid pool, i.e. precursor + free amino acids → enzyme. Although *E. coli* growing in mineral salt medium can clearly synthesize all the amino acids it requires to form protein, the amino acid pool is relatively small (Taylor, 1947) and the enzyme system may possibly not be activated unless preformed amino acids are supplied externally. Some of the present findings offer a parallel to those of other workers concerning the formic hydrogenlyase of *E. coli* (Lascelles 1948; Billen & Lichstein, 1951; Pinsky & Stokes, 1952). The speculation may be made that for citridesmolase formation in *E. coli* the above reaction proceeds rapidly from left to right only in the presence of glucose as energy source and that when this is no longer available, and particularly when cells are placed in a mineral salt medium, there is a readjustment in the reverse direction as the concentrations in the free amino acid pool decrease. Our results suggest, but do not establish, that enzyme precursor may be formed during growth on glucose in mineral salt medium containing citrate, since the maximum activity acquired by these cells for a given amount of glucose present in the peptone + citrate + glucose mixture is greater than when citrate is omitted from the mineral salt medium (Table 2). Alternatively, the former cells may become permeable to citrate during growth, thereby permitting a more rapid development of enzyme in the activation test. Immediate activation was indeed found, whereas cells grown in the absence of citrate showed a lag period, but without postulating precursor formation it is difficult to account for the greater amounts of enzyme given per unit quantity of glucose in one case than the other. Further, the data for disintegrated cells appear to exclude a mechanism based on cell-permeability as the cause of differences in activity between different batches of *E. coli.* In contrasting

E. coli with *Aerobacter aerogenes* it may also be relevant to observe that not only is the latter able to grow in a citrate + mineral salt medium but also develops therein strong formic hydrogenlyase without addition of preformed amino acids (Dagley & Dawes, 1953*a*).

It is a pleasure to acknowledge the skilful technical assistance of Mrs M. Lees and the co-operation of Dr A. R. Johnson in the early stages of the work.

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pH Stability and Purification of Lumpy Skin Disease Virus

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SUMMARY: Lumpy skin disease virus in allantoic fluid was found to be stable when subjected to wide variation of hydrogen-ion concentration under differing conditions of time and temperature. The virus could be concentrated by adsorption on the precipitate formed when allantoic fluid was dialysed at pH 4·5; adsorption on calcium phosphate was also demonstrated. Preliminary purification of the virus could be effected by these methods.

The isolation of a filtrable agent from a calf dead of lumpy skin disease (LSD) was reported by van den Ende, Don & Kipps (1949). In a later paper van den Ende & Turner (1950) described a method of titrating the virus and applied it in neutralization tests and in the determination of certain physical properties. The data reported in this communication are from continued observations on the properties and behaviour of the virus.

MATERIAL AND METHODS

Virus suspensions. These consisted of allantoic fluids from Leghorn chicken eggs inoculated on the 9th day of incubation and harvested after 3–4 days of further incubation at 37°. The eggs were chilled before harvesting to obtain fluids free from blood. The pooled fluids were either used immediately or stored at 0–4° until they were required. Material stored over long periods was kept at $-70°$.

Buffer solutions. For the pH range 2·0–8·0 McIlvaine's standard 0·1M-citric acid + 0·2M-disodium phosphate mixtures were used. For pH 10·0, 0·1M-glycine or 0·1M-disodium phosphate adjusted with 0·1M-sodium hydroxide was used. For pH 12·0, 0·1M-disodium phosphate was similarly adjusted with 0·1M-sodium hydroxide. pH 1·0 'buffer' consisted of 0·1M-hydrochloric acid.

Diluent. Dilutions for virus titrations were made in nutrient broth of pH 7·0.

Virus titrations. Tenfold serial dilutions were made in broth and 0·2 ml. amounts of the dilutions were inoculated sub-allantoically into 9-day Leghorn chick embryos. At least five eggs were used for each dilution. The eggs were 'candled' daily for 10 days and times of death recorded. Deaths which occurred on the 1st day after inoculation were regarded as being non-specific. LD 50 end-points were calculated by the method of Reed & Muench (1938). All inocula were tested for bacterial sterility on blood agar slopes. All solutions were prepared in distilled water and sterilized by filtration through Seitz pads where necessary. The pH values of buffer solutions were checked by glass electrode measurements. Penicillin and streptomycin were added in manipulations where a high probability of bacterial contamination was considered likely.

RESULTS

Stability at different pH *values.* Volumes (10 ml.) of buffer solutions of pH values 1·0, 2·0, 4·0, 6·0, 8·0, 10·0 and 12·0 were tubed in duplicate. To each tube was added 1·0 ml. of virus-containing allantoic fluid. The tubes were then held at 37° in a water-bath or in the refrigerator at 0–4° for various times. When the time periods had elapsed one series of tubes was used for a determination of pH value and the individual tubes of the other series were immediately adjusted to pH 7·0. Disodium phosphate or citric acid were used for neutralization of the pH 2·0–8·0 series and 0·1 M-sodium hydroxide or 0·1 M-hydrochloric acid for the remainder. The volume of each mixture was brought to 100 ml. with pH 7·0 buffer, thus giving a dilution of 1/100 with respect to the initial amount of virus added. Further tenfold dilutions were made from each of these samples and titrated in eggs. A control titration of untreated starting material was included with each experiment. The pH value of this initial allantoic fluid was 7·6. The pH values of the mixtures showed little variation during the time of the exposure except that the pH values of the samples at the extremes of the range had altered. The sample originally at pH 10 had fallen to pH 8·9 and that at pH 12·0 to 11·8; the samples at pH 1·0 and 2·0 originally, increased to 1·2 and 2·3 respectively.

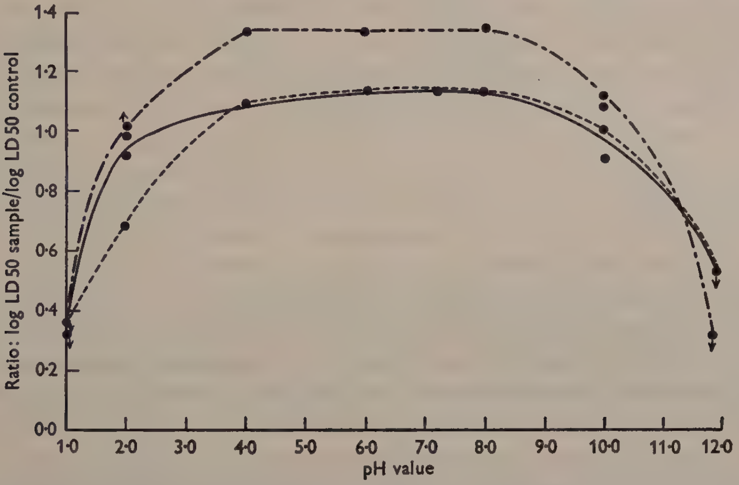

Fig. 1. Graph showing the stability of lumpy skin disease virus exposed to buffers of various pH values. —-—, 14 days at 0–4°; - - - -, 4 hr. at 37°; ——, 1 hr. at 37°.

The results have been recorded graphically in Fig. 1 and are expressed as ratios of $\log_{10}$ LD 50 titre of the exposed sample to $\log_{10}$ LD 50 titre of the untreated control. It will be seen that the virus infectivity remained relatively stable when it was subjected to wide variations in pH value of the suspending fluid. For 1 hr. at 37° and 14 days at 0–4° the virus was stable at values from pH 2·0 to 10·0. Diminution of activity was apparent at pH 2·0 after 4 hr. exposure at 37°, and there was rapid inactivation at pH's 1·0 and 11·8 under all the tested conditions of time and temperature. It is of interest that over the range at which the virus remains stable the samples held diluted in buffer had higher titres than the control material.

Precipitation of the virus with a protein fraction of allantoic fluid

It was noted during the pH experiments that a precipitate occurred when allantoic fluid was exposed to a hydrogen-ion concentration of pH 4·0. Both infected and normal allantoic fluid behaved similarly in this respect, although the precipitate appeared to be heavier in the infected allantoic fluid. In preliminary experiments allantoic fluid was adjusted to *c.* pH 4·8 by the direct addition of 0·1 N-HCl. The bright yellow precipitate was redissolved in its original volume of 0·07 M-Na_2HPO_4 buffer at pH 8·2 and had a higher virus content than its supernatant fluid. The nature of the precipitate was examined electrophoretically. Samples of infected allantoic fluid (50 ml.) and of normal allantoic fluid (50 ml.) harvested from eggs of the same age were dialysed in viscose sausage casing bags against 2 l. citric phosphate buffer (pH 4·0) at 0–4° for 48 hr. This provided a mild means of changing the pH value of the fluids; the precipitates obtained were possibly heavier. The precipitates were recovered by centrifugation and each was redissolved in 7·5 ml. of pH 8·2 0·07 M-phosphate buffer. The solutions were dialysed overnight against 0·013 M-phosphate buffer (pH 8·2) containing 0·044 M-NaCl. After centrifugation at 10,000 r.p.m. for 15 min. the solutions remained slightly opalescent. They were then examined electrophoretically using a double cell (Polson, 1952) under a potential gradient of 5 V./cm. The material from the infected allantoic fluid showed no evidence of inhomogeneity and migrated as one boundary. It contained approximately 10 times as much protein as the material from normal fluid. Tryptic digestion followed by ultracentrifugation in both cases yielded surface layers of lipid, indicating that the precipitates were lipo-protein in nature. Later experiments, however, showed that lipid was neither a constant nor necessary factor in 'virus precipitation'.

The preliminary experiment quoted above indicated that protein precipitates might carry with them a large proportion of the virus content of infected allantoic fluid and could be utilized in the concentration and purification of the virus.

The pH value for optimum virus precipitation

Experiments were set up to determine the pH value at which maximal precipitation of virus activity occurred. Volumes (20 ml.) of infected allantoic fluid in viscose sausage casing were dialysed for 2 days at 0–4° against 1 l. volumes of McIlvaine's citric phosphate buffer at pH values 3·2, 3·6, 4·0 and 4·8. The precipitates obtained were recovered by centrifuging at 3000 r.p.m. for 30 min., and dissolved in 20 ml. (the original volume) of 0·07 M-phosphate buffer pH 8·2. These solutions, together with their respective supernatant fluids, were then titrated in eggs. Controls to these titrations consisted of the original untreated allantoic fluid. It will be seen from Fig. 2 that over the pH range 3·2–4·4 there was approximately 100 times more virus in the precipitates than in their respective supernatants and an optimum is indicated between pH 4·0 and 4·4. At pH 4·8 only scanty precipitation occurred and, as the figure shows, almost identical titres were obtained for resuspended precipitate and supernatant fluid. The titres of precipitates obtained at pH 3·2 were still high, but in this region and below, pH values were approached at which the virus becomes unstable.

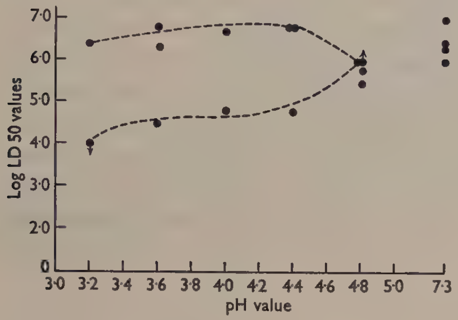

Fig. 2

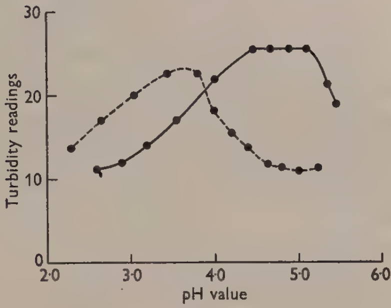

Fig. 3

Fig. 2. Titres (expressed in neg. log. LD 50) of the precipitates and supernatant fluid obtained from allantoic fluid dialysed against buffers of various pH values. Top curve = virus titres in precipitates. Bottom curve = virus titres in corresponding supernatant fluids. The values at pH 7·3 are those of the original untreated alantoic fluids.

Fig. 3. Turbidity readings for normal allantoic fluid diluted in McIlvaine's buffer at different pH values. Broken curve with standard buffer concentration; solid curve with buffer diluted 1/5 in distilled water.

Decrease of the salt concentration of the buffers used in the precipitation of the virus resulted in the production of heavier precipitates. In a preliminary experiment McIlvaine's buffer mixtures in 10 ml. volumes were prepared to cover a range from approximately pH 2·0 to 5·5, one series having the normal salt concentration and the other one-fifth of this amount. Samples (0·25 ml.) of infected allantoic fluid were added to each tube and the turbidities compared in a Klett colorimeter before coagulation had occurred. Water was used as a standard. Absolute values of optical density or light transmission were not calculated but colorimeter readings were directly plotted against pH values. Fig. 3 shows that with more dilute buffer a heavier precipitate was obtained and that the pH range at which optimal precipitation occurred was both extended and shifted to a slightly more alkaline level. Subsequent experiments were done with precipitates obtained by dialysis of allantoic fluid against McIlvaine's citric phosphate mixtures diluted 1/5 and having a pH value of 4·5.

It had been hoped that some purification could be effected by preferentially precipitating contaminating protein at one salt concentration and virus at another. Virus activity accompanied the heaviest precipitate and it seemed probable that the precise nature of the protein precipitated was not a decisive factor in the 'precipitation' of the virus, the bulky protein precipitates behaving as adsorbents and carrying the virus with them. Confirmation of this was obtained in an experiment where the virus from 300 ml. infected allantoic fluid was concentrated by precipitation and partially purified by ether extraction and ultracentrifugation. When a protein solution, obtained from normal allantoic fluid, was added to this material it yielded on dialysis at pH 4·5 a precipitate which again carried with it the bulk of the virus. Some evidence was obtained that calcium phosphate could act as an adsorbent for LSD virus. In a typical experiment calcium phosphate was precipitated in the presence of allantoic fluid after the method described by Herbert & Todd (1941). Samples (4·0 ml.) of infected allantoic fluid were each mixed with 4·5 ml. 0·066 M-disodium hydrogen phosphate followed by 0·36 ml. of 1·0 M-calcium acetate. Precipitation was allowed to occur at room temperature for 20 min. The precipitates were collected by centrifuging and either immediately resuspended in broth to the original volume (4·0 ml.) or washed twice in 5·0 ml. saline and then resuspended. The washed and unwashed precipitates after resuspension together with the supernatant fluid were titrated in eggs. These results are recorded in Table 1.

Table 1. *Titrations showing adsorption of lumpy skin disease virus on calcium phosphate precipitate*

	Dilution of sample					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
Sample	Eggs dead/eggs inoculated					LD 50
Initial allantoic fluid	—	—	—	8/8	—	$>10^{5\cdot0}$
Supernatant after precipitation	7/12	—	1/10	—	1/11	$10^{2\cdot5}$
Resuspended precipitate	11/12	—	10/12	—	4/12	$10^{5\cdot2}$
Resuspended precipitate twice washed	5/5	—	5/6	—	1/6	$10^{5\cdot0}$
Control calcium phosphate precipitate	2/8	—	—	—	—	$<10^{2\cdot0}$

Subsequent removal of the protein from redissolved virus-protein precipitates was attempted in various ways.

Tryptic digestion. Preliminary experiments indicated that the virus was stable when treated with crystalline trypsin in a concentration of 0·033 % for periods up to 150 min. In a typical experiment 700 ml. infected allantoic fluid was dialysed against two changes of 10 l. of pH 4·5 McIlvaine buffer diluted 1/5 with distilled water. After 48 hr. the very heavy precipitate within the cellophan was collected by centrifuging at 2000 r.p.m. for 1 hr. This precipitate was dissolved in 35 ml. 0·2 M-phosphate buffer (pH 8·2). This solution was extracted with an equal volume of ether at 0° and centrifuged for 1 hr. at 2000 r.p.m. in a refrigerated centrifuge. No lipo-protein precipitate was present at the ether/water interface after centrifuging but the ether layer was yellow in colour. The ether layer was removed. A deposit of some crystalline material below the aqueous layer was extracted with 5 ml. saline and added to the aqueous layer. The ether extraction was repeated and residual dissolved ether removed from the aqueous phase by evaporation at a water pump. The aqueous phase was then centrifuged at 20,000 r.p.m. for 15 min. in a Spinco Model L preparative centrifuge and the deposit discarded. The supernatant aqueous solution was then again centrifuged, this time at 30,000 r.p.m. for 2 hr. The pellet so obtained was dispersed in 20 ml. 0·07M-phosphate buffer and treated with 0·002 g. of crystalline trypsin for 1 hr. at 37°. The digested material was centrifuged at 30,000 r.p.m. for 2 hr. and yielded a pellet of volume 6–7 mm^3. which was redispersed in 1 ml. 0·07M-buffer at pH 8·2. Samples of the original allantoic fluid and the final concentrate were titrated in eggs. The titres (LD 50) of the original and concentrated material were 5·6 and 8·2 respectively, indicating a concentration of approximately 400 times, i.e. 40 % of the total infective virus was recovered.

Chloroform extraction of the virus-protein precipitate. The precipitate obtained from 100 ml. infected allantoic fluid dialysed at pH 4·5 as described previously was redissolved in 15 ml. of 0·07M-phosphate buffer (pH 8·2). The solution was mechanically shaken for 7 min. in an ice bath with an equal volume of chloroform containing 5·0 % (w/v) amyl alcohol. The mixture was centrifuged for 15 min. at 3000 r.p.m. in a refrigerated centrifuge and the chloroform together with the heavy precipitate at the chloroform/aqueous interface discarded. The chloroform extraction was repeated, giving a total time of contact of 30 min. A second layer of precipitate at the liquid/liquid interface was discarded after centrifuging in the same way.

The aqueous layer was freed from chloroform under reduced pressure. Some odour of amyl alcohol was still apparent in the water-clear fluid, which gave no precipitate when tested with trichloroacetic acid. The fluid made up to its original volume (100 ml.), as well as a sample of the original allantoic fluid, was titrated in eggs. The log. LD 50 titres of original and chloroform-extracted material were 6·3 and 5·7 respectively. While there was therefore a decrease in titre the removal of large amounts of protein justified the use of chloroform in further experiments.

In another experiment 550 ml. virus-infected allantoic fluid were dialysed at pH 4·5 as before. The precipitate obtained was redissolved in 25 ml. 0·07M-phosphate buffer (pH 8·2) and centrifuged at 30,000 r.p.m. for 2 hr. The pellets were dispersed in 10 ml. pH 8·2 buffer and subjected to six successive extractions with chloroform + amyl alcohol mixture. The extractions were done at 0° and occupied a total time of 90 min. A water-clear protein-free fluid was obtained. A sample of this material was diluted to be comparable with the original allantoic fluid and titrated in eggs. The log. LD 50 titres of original and chloroform treated material were respectively 6·4 and $<4·0$. From these results it would appear that while chloroform was an admirable means of removing protein the drastic extractions described has a lethal effect on the virus or the virus remained adsorbed on the chloroform protein precipitate and was lost. Although a large part of the infectivity of the material was lost, on examination in the spectrophotometer it showed absorption in the range usually associated with nucleoprotein. No such absorption was observed when similarly processed material from normal allantoic fluid was examined.

DISCUSSION

The finding that lumpy skin disease virus is stable over a wide range of hydrogen-ion concentration provides information of use for further purification studies. Similar behaviour is exhibited by a number of other viruses. Under certain conditions of time and temperature, vaccinia (Beard, Finkelstein & Wyckoff, 1938), influenza (Stock & Francis, 1940), mumps (Weil, Beard, Sharp & Beard, 1948) and Lansing poliomyelitis virus (Bachrach & Schwerdt, 1952) all retained their infectivity when exposed to wide ranges of hydrogen-ion concentration. On the other hand, Alexander (1935) showed the extreme sensitivity of horsesickness virus which, while stable to a range of values above pH 5·96, underwent immediate inactivation when exposed to pH values even slightly less than this value.

Protein precipitation in the concentration of virus suspensions is not a novel process. Theiler & Gard (1940) with mouse encephalomyelitis showed that 90 % of virus activity was found in the floccules which formed when mouse brain suspensions were adjusted to pH 4·5. The iso-electric precipitation of proteins from brain suspensions of virus has been utilized in concentrating strains of human poliomyelitis virus (Bourdillon & Moore, 1942; Herrarte & Francis, 1943; Bachrach & Schwerdt, 1952), the pH value for precipitation in each case being 4·6. It is significant that the proteins in normal allantoic fluid were precipitated at almost identical pH values and could be similarly utilized to concentrate the LSD virus.

Salk (1941) and Stanley (1945) used calcium phosphate adsorption techniques in the concentration of influenza virus. Similar procedures have been used here, and no claim is made for the selectivity of calcium phosphate as a specific adsorbent for LSD virus. Both precipitation procedures show that LSD virus shares a property of 'adsorbability' with other viruses, which can be utilized in effecting initial concentration.

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Effects of a Gene Mutation in *Neurospora crassa* relating to Glutamic Dehydrogenase Formation

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SUMMARY: Two strains of *Neurospora crassa*, which require α-amino nitrogen for growth as a result of a single gene mutation (*am*), lack glutamic dehydrogenase. Extracts of mutant mycelium contained no detectable enzyme, although as little as 0·2 % of the amount normally present in the wild type should have been detectable in some experiments. Mutant extracts contained no detectable enzyme inhibitor. A balanced heterocaryon containing *am* nuclei and nuclei carrying an unrelated mutation produced less enzyme than did wild type mycelium, but there was no indication that the *am* nuclei were suppressing the activity of the non-*am* nuclei in promoting enzyme production. The production of the enzyme by the wild type was not strikingly dependent on conditions of growth although the addition of glutamate to the medium seemed somewhat to depress the enzyme concentration. This effect was more marked when glutamate was supplied as a sole nitrogen source. The mutation had little or no effect on the concentrations of two other dehydrogenases or of several transaminases, all related to glutamic dehydrogenase in their substrate specificities. The possible significance of these observations is discussed.

A previous paper (Fincham, 1950) reported on mutant strains of *Neurospora crassa* (No. 32213 and 47305) which had a nutritional requirement for α-amino nitrogen for growth without a prolonged lag. It was subsequently found (Fincham, 1951*a*) that these mutants apparently lacked glutamic dehydrogenase. Two derivatives of each mutant did not yield detectable amounts of this enzyme in repeated trials, while a number of wild-type strains and mutants of other kinds all gave active enzyme preparations. The fact that any one of a number of different α-amino acids will replace glutamic acid in supporting growth is satisfactorily accounted for by the presence of a range of transaminases in *N. crassa* (Fincham, 1951*b*).

The experiments reported in this paper were designed to test the previous conclusions by more quantitative measurements of enzyme activity and to gain further information as to the nature of the gene/enzyme relationship.

METHODS

Chemicals. α-Ketoglutaric acid (purchased from British Drug Houses Ltd. or from L. Light and Co.) was recrystallized before use from acetone + benzene (Umbreit, Burris & Stauffer, 1949). DL-Isocitric acid was obtained from L. Light and Co. and hexose-6-phosphate (*c.* 70 % glucose- and 30 % fructose-6-phosphate) was a gift from Dr B. C. Loughman. Preparations containing coenzyme II (triphosphopyridine nucleotide = TPN) were made from liver according to the method of LePage & Mueller (1949) up to the stage of chromatographic separation of TPN from DPN. Preparations from beef, pig and sheep liver gave products containing 49, 36 and 31 % TPN respectively;

the sheep liver preparation was the one used in most of the experiments reported here. Reduced coenzyme II (TPNH) was prepared by reduction with isocitrate and pig heart extract containing isocitric dehydrogenase according to the method of Evans & Nason (1953), with the modifications that only the empirically determined minimum amount of isocitrate necessary to reduce the TPN was used, and that it was not found necessary to add magnesium ions to obtain adequate activity of the pig heart enzyme. The phosphate buffer referred to throughout this paper was 0·067 M phosphate at pH 7·4 (4 parts Na_2HPO_4:1 part KH_2PO_4, B.D.H. Analar grade, in glass-distilled water).

Neurospora *strains.* The two wild-type strains used in most experiments were 126–1 and 133–2, both of mating-type *A*. These strains, which were used because of their vigour and high fertility, were isolated from experimental crosses in this laboratory by Mr J. A. Pateman. The mutant strain which was used in all the experiments reported here was an *albino* re-isolate, derived originally from strain 32213, of mating-type *a* (isolation number 1111–78). This strain will be referred by the gene symbol *am* (amino-requiring). Two derivatives of *am* which had recovered the ability to grow normally on minimal medium following ultraviolet irradiation were obtained by Mr Pateman, and were designated UV 17 and UV 21. UV 17 proved to be a heterocaryon containing unchanged *am* nuclei, and nuclei which were wild-type with respect to the *am* locus but which had acquired an additional mutation resulting in a requirement for cytidine. Uridine, which would most probably also have been active, was not tested. The new mutation will be given the gene symbol *pyr* (pyrimidine-requiring). The *am* and *pyr* components of the heterocaryon were obtained in homocaryons by isolation of single conidia. UV 21 was also heterocaryotic, containing unchanged *am* nuclei, when it was first isolated following irradiation, but it yielded a homocaryotic wild-type back-mutant strain (designated UV 21-W) following isolation of single conidia. The genetic data relating to these back-mutations will be published elsewhere.

Media. For growth of mycelium for enzyme extractions 150 ml. lots of liquid medium in bottles giving a depth of medium of *c.* 1 cm. were used. The usual minimal medium (Fries no. 3) contained, per litre, 5 g. ammonium tartrate; 1 g. NH_4NO_3; 1 g. KH_2PO_4; 0·5 g. $MgSO_4.7H_2O$; 0·1 g. $CaCl_2$; 0·1 g. NaCl; 15 g. sucrose; 5 μg. D-biotin; trace elements (Ryan, Beadle & Tatum, 1943). In experiments where it was desired to vary the nitrogen source without causing wide changes in pH value a basal N-free buffered medium was used containing, per litre: 5 g. potassium tartrate; 6·6 g. KH_2PO_4 + 0·28 g. Na_2HPO_4 (giving 0·067 M-phosphate, pH 5·6); $MgSO_4$, $CaCl_2$, NaCl, trace elements, sucrose and biotin as in ordinary minimal medium. Stock cultures were kept on 1·5 % agar slopes of appropriately supplemented minimal medium.

Preparation of extracts. Mycelium was grown for 48 hr. at 25° without agitation from an inoculum consisting of 0·5–1 ml./bottle of a distinctly turbid suspension of 2- to 4-day old conidia. After washing and pressing out excess moisture (Fincham, 1951 *a*) the mycelia usually weighed 1·0–2·5 g. Extraction was carried out by the grinding and filtration procedure previously described

(Fincham, 1951*a*) or, more conveniently and in all experiments unless otherwise stated, by homogenizing with 100 ml. phosphate buffer (pre-chilled to 0–5°) for 90 sec. in the monel metal container of an M.S.E. 'Atomix' blender, followed by filtration by suction through a washed pad of 6 g. kieselguhr supported on a filter-paper on a 6 cm. diameter Buchner funnel. Extracts so obtained were free from microscopically visible particles and contained 0·2–0·5 mg. protein/ml.

Protein determinations. Protein of extracts was determined by the following method, based on Stickland (1951). To 5 ml. of extract in phosphate buffer, 0·9 ml. of 20 % (w/v) NaOH and then 0·15 ml. 25 % (w/v) $CuSO_4.5H_2O$ was added. The precipitate was broken up with a glass rod and centrifuged after standing for 5 min. The colour in the supernatant was read in an 'Eel' photoelectric colorimeter with a green filter, and was proportional to protein up to at least 1 mg. protein/ml., subject to an error of *c.* 0·03 mg. protein/ml. Determinations were made in quadruplicate and averaged. The method was standardized against casein and against a preparation of neurospora protein. The colour intensity/mg. protein was the same in both cases.

Enzyme assay procedures. Glutamic dehydrogenase was assayed by following oxidation of TPNH in a Unicam quartz spectrophotometer cell. To a 1 cm. cell was added 1 ml. mycelial extract, 5 μmole (or 6·7 μmole in Expts. 4 and 5, Tables 1 and 2) Na α-ketoglutarate, 10 μmole NH_4Cl, and 1·85 ml. phosphate buffer to a total volume of 3 ml. The density at wavelength 340 mμ. was read, and at zero time 0·2 ml. of a solution of TPNH was added to give an initial increase in optical density of *c.* 0·40, corresponding to 3×10^{-5} M-TPNH. The optical density was read at 1 min. intervals. The rate of reoxidation of TPNH was constant down to an E_{340} of *c.* 0·25, and was proportional to enzyme concentration (Fig. 1). Enzyme activity was expressed as change in E_{340}/min./mg. protein $\times 10^3$. Assays were performed in batches of three, and different batches were not always directly comparable owing to variations in room temperature. By repeating one assay from each batch in the subsequent run, and assuming the same Q_{10} for every digest, assays within a single experiment were made comparable by the use of correction factors generally of the order of 5–15 %. Values for enzyme activities from different experiments may not, however, be closely comparable. Room temperatures varied between 17 and 20°.

In some earlier experiments, some of the results of which are referred to in the text, use was made of the fact that neurospora extracts will reduce TPN even when no reducing substrate is added. This is almost certainly due, not to oxidation of an impurity in the coenzyme as previously supposed (Fincham, 1951*a*), but to non-dialysable polysaccharide present in the extracts (Tissiéres, Mitchell & Haskins, 1953). TPN was added to mycelial extract in a spectrophotometer cell, the reduction of TPN followed until the concentration of TPNH remained constant, and then α-ketoglutarate and NH_4Cl were added and the reoxidation rate measured. The disadvantage of this method was that different extracts reduced TPN to different degrees; in some cases the TPN was completely reduced and enough endogenous substrate remained to hinder the reoxidation reaction, while in others the TPNH level attained was insufficient for reoxidation to proceed at the maximum rate. The method proved useful as a simple and sensitive qualitative test for the presence or absence of enzyme, and served in favourable instances for quantitative comparisons.

All additions to reaction mixtures were thoroughly mixed in by immediately inverting the cell. Some of the small fluctuations in optical density previously observed following additions to inactive extracts (Fincham, 1951*a*) were probably due to inadequate mixing.

Other determinations. Ammonia was determined by microdistillation followed by nesslerization (Fincham, 1951*a*). Glutamic acid was determined in the experiments on transaminases by the method of Fowden (1951), digests being concentrated to one-quarter of their original volume and applied to Whatman no. 3 paper chromatograms as two superimposed 5 μl. spots.

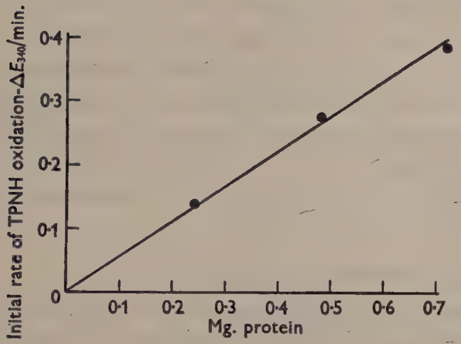

Fig. 1

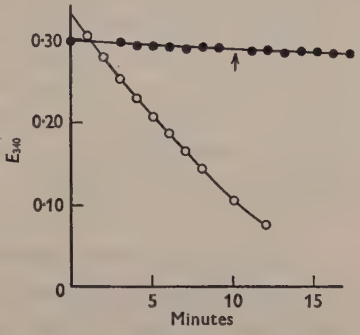

Fig. 2

Fig. 1. Relation between enzyme activity and concentration of protein of extract of wild-type grown on N-free medium +0·01 N ammonium tartrate. Data from Expt. 1 (cf. Table 1). Details of assay procedure in the text.

Fig. 2. Reoxidation of TPNH in the standard glutamic dehydrogenase assay system by extract of *pyr* mycelium, ○—○, containing 0·25 mg. protein, compared with the result using extract of *am* mycelium, ●—●, containing 0·90 mg. protein. Data from Expts. 4 and 3 respectively (cf. Table 2). In the experiment with *pyr* α-ketoglutarate and NH_4Cl were present at time 0; in the experiment on *am* these substrates were added at 10 min. and subsequent readings are corrected for change in vol. (3·2→3·35 ml.) and for α-ketoglutarate absorption.

RESULTS

Absence of enzyme in the mutant

Fig. 2 shows the results of typical enzyme assays on extracts of mutant and non-mutant mycelium. A very slow decrease in density following addition of TPNH was often observed in experiments on mutant extracts, but this proceeded (Fig. 2) at a rate independent of the presence of α-ketoglutarate and ammonia, and was presumably due to a slow destruction of TPNH which had nothing to do with the glutamic dehydrogenase system. This breakdown of TPNH was so slow that it was neglected in computing enzyme activities. In the experiment shown in Fig. 2, 1 % of normal activity in the mutant extract would have been detectable. In other experiments where higher protein concentrations were used, more than 0·2 % of normal activity in the mutant extract appeared to be excluded.

It has been confirmed that mutant mycelium which had, after a prolonged lag period, acquired a phenotypic adaptation to growth on minimal medium still yielded no detectable glutamic dehydrogenase. The same applies to mutant mycelium which had been grown on minimal medium with the addition of 0·005 M-glutamate. This small concentration of amino acid allowed growth to start without lag and rapidly disappeared from the medium, leaving the mutant mycelium growing solely on inorganic nitrogen. The question of the mechanism of ammonia assimilation under these conditions is an interesting one which has not yet been answered.

Absence of an enzyme inhibitor from mutant extracts

It was previously reported (Fincham, 1951 *a*) that there was no pronounced depression of enzyme activity when mutant extract was added to wild-type extract. This experiment was repeated and the enzyme activities determined more quantitatively. The activity of wild-type extract with and without the addition of an equivalent amount (in terms of protein) of mutant extract was the same to within 3 %. It seems clear that the absence of activity of mutant extracts cannot be explained by the presence of a dissociable enzyme inhibitor.

Effect of growth conditions on the activity of wild-type extracts

A complication which arose in comparing wild type and mutant was that it was difficult to grow the two under identical conditions. The mutant took up ammonia more slowly than the wild type and consequently, in media containing ammonium salts or nitrates (which are reduced to ammonia by neurospora mycelium), the pH value tended to be higher in cultures of the mutant than with wild type. Thus it was of interest to know how sensitive glutamic dehydrogenase production in the wild type was to alterations in the composition of the medium.

The data shown in Table 1 indicate that the production of the enzyme by wild-type mycelium was not markedly dependent on conditions of growth. Even where glutamic acid was supplied as sole source of nitrogen, under which conditions one might expect glutamic dehydrogenase to be an easily dispensable enzyme and conditions of growth for wild type and mutant should be as closely similar as possible, the concentration of enzyme was still more than 50 % of that found when ammonia was the main nitrogen source in Expts. 4 and 5, although a more striking effect of altering the nitrogen source had been found in Expt. 1. Thus the production of glutamic dehydrogenase is not dependent on the presence of inorganic nitrogen in the medium. The addition of 0·01 M-glutamate to the medium had some effect in decreasing the concentration of glutamic dehydrogenase, the decrease being of the order of 30 %. This decrease was more marked when glutamic acid was sole nitrogen source. The effect of glutamate on the concentration of glutamic dehydrogenase might provide an explanation for the sparing effect of this substance on ammonia uptake (Table 1), which has been observed in a number of experiments apart from those reported here. Possibly parallel instances of a decrease of enzyme concentration in bacteria by the addition to the growth medium of the product of the reaction catalysed by the enzyme in question have been reported by Cohn & Monod (1953).

Table 1. *Production of glutamic dehydrogenase by the wild type grown with various sources of nitrogen*

All nitrogen sources were at 0·01 M (or 0·01 N in case of NH_4 tartrate) except the second NH_4 tartrate addition in Expt. 5, which was 0·02 N.

Expt.	Nitrogen source	Wet wt. (g.)	Final pH	Ammonia used (%)	Enzyme activity
1	KNO_3	1·3	6·35	—	55
	NH_4 tartrate	1·9	4·55	—	69
	Glutamic acid*	1·5	5·50	—	13
4	NH_4 tartrate	1·5	4·50	93	74
	NH_4 tartrate + glutamic acid*	2·1	5·10	75	50
	Glutamic acid*	1·3	5·95	—	43
5	NH_4 tartrate	1·5	4·45	93	121
	NH_4 tartrate	1·55	4·42	87	107
	NH_4 tartrate + glutamic acid*	2·55	4·90	89	82
	NH_4 tartrate + glutamic acid	2·0	4·66	85	60
	Glutamic acid*	1·35	5·45	—	75
	Glutamic acid	0·6	4·55	—	45

* Half-neutralized with NaOH.

Production of glutamic dehydrogenase by heterocaryons

It seemed possible that more information concerning the relation between production of the enzyme and the gene mutation could be obtained by studying enzyme production by heterocaryons carrying varying proportions of *am* nuclei. With this end in view the heterocaryon UV 17 (*am* + *pyr*), which grows like the wild type, was grown on minimal medium, and on minimal medium supplemented with cytidine or glutamate, and the glutamic dehydrogenase activities were compared with the activities in the two homocaryotic derivatives by isolation of single conidia.

To ensure that the mycelium being studied was heterocaryotic, UV 17 cultures were always started from inocula consisting of 0·5 ml. of a coarse blend of mycelium prepared as follows. UV 17 conidia were plated on minimal agar and single germinating conidia which showed the wild phenotype were used to inoculate 50 ml. minimal medium. After 2–3 days at 25° the resulting mycelium was washed with sterile distilled water and blended for 30 sec. at half-speed in 100 ml. sterile distilled water in a sterilized blender container. If unselected conidia from UV 17 cultures had been used as inoculum there would have been danger of overgrowth by one or other type of homocaryon in the supplemented media.

Nuclear ratios in UV 17 cultures were estimated by the following procedure.

Pads of mycelium ready for enzyme extraction were washed in sterile distilled water, blotted and blended in 100 ml. sterile buffer in the sterile blender container at half-speed for 25 sec.; 0·75 ml. of the coarse blend was then withdrawn aseptically and run on to a 6-day culture of wild type of mating type *A* growing on a slope of Westergaard & Mitchell's (1947) crossing medium. The rest of the blend was then further blended for 80 sec. at full speed and filtered to be used for enzyme assay in the usual way. The fertilized cultures discharged ascospores on to the inside of the tube in about 2 weeks. Ascospores were collected from as wide an area as possible, spread on plates of minimal agar, activated by heating for 30 min. at 60°, and left for 16 hr. at room temperature. After this time germinating wild-type ascospores with long branching hyphae could be clearly distinguished from *pyr* spores with very short almost spherical germ tubes, and distinguished with fair certainty from *am* spores which had thin tapering hyphae of medium length. The two mutant types combined were always essentially equal in number to wild-type ascospores, indicating that there was no differential germination. At least 500 ascospores were counted for each determination. The true values of the ascospore ratio from which the estimated ratio would be just significantly different at the 5 % level can be calculated by means of the χ^2 method, and the resulting confidence limits are approximately indicated in Table 2. The actual likely error in the estimate of the nuclear ratio will be greater than thus indicated, since the ascospore ratio is itself only an estimate of the nuclear ratio subject to a sampling error depending on the number of perithecia (probably usually of the order of a few hundred) from which ascospores have been taken. The method did, however, give reasonable results in a reconstruction experiment in which known proportions of *am* and *pyr* homocaryotic mycelia were blended together. Blends containing, on a dry weight basis, 20, 57 and 87 % of *pyr* mycelium gave estimated percentages of *pyr* nuclei of 15, 56 and 84 respectively.

The results of the experiments on the heterocaryon are given in Table 2. Extracts of heterocaryotic mycelium gave consistently less enzyme activity than extracts of the derived *pyr* homocaryon, which itself gave about the same activity as wild type (it has been shown that the supplementation of minimal medium with cytidine has little or no effect on enzyme level in the wild type). The proportion of normal activity shown by the heterocaryon was, in general, of the same order as the proportion of *pyr* nuclei, or somewhat greater. In Expt. 4 (Table 2) the concentration of glutamic dehydrogenase was markedly depressed by the supplementation of the medium with glutamate, and a similar, though less striking effect, was shown in Expts. 2 and 3. As already noted, glutamate had a similar effect on the wild type, and the rather larger depression of enzyme level in UV17 in Expt. 4 may have been due to the same cause, perhaps combined with an increase in the proportion of *am* nuclei in the presence of glutamate. Any such shift in the nuclear ratio was evidently quite small; in fact the nuclear ratio in this heterocaryon in the experiments as a whole showed surprising stability to changes in the composition of the medium.

The data in Tables 1 and 2 suggest that higher yields of glutamic dehydrogenase were obtained from mycelium grown on the N-free medium supplemented with 0·01 N-ammonium salt than from mycelium grown on the ordinary minimal medium, though the high activities in Expts. 4 and 5 are partly attributable to the slightly higher α-ketoglutarate concentration adopted in the assay system in these experiments.

Table 2. *Production of glutamic dehydrogenase by the heterocaryon UV17 and derived homocaryons*

Expt. no.	Strain	Medium	Wet wt. (g.)	Enzyme activity	Estimated % *am*$^+$ nuclei
2, 3	*pyr*	Minimal + 0·15 mg./ml. cytidine	1·55, 1·5	49, 36	100
	UV17	Minimal	1·15, 1·6	32, 24	54 ± 8, 57 ± 7
	UV17	Minimal + 0·01 M-glutamic acid*	1·75, 1·95	23, 14	70 ± 6, 50 ± 7
	UV17	Minimal + 0·15 mg./ml. cytidine	1·55, 1·5	32, 33	71 ± 8, 44 ± 5
	am	Minimal + 0·01 M-glutamic acid*	1·1, 1·5	0, 0	0
4	*pyr*	N-free + 0·15 mg./ml. cytidine + 0·01 N-NH_4 tartrate	1·6 † 1·6	81 91	100
	UV17	N-free + 0·01 N-NH_4 tartrate	1·3 1·6	64 66	70 ± 6‡
	UV17	N-free + 0·01 N-NH_4 tartrate + 0·01 M-glutamic acid*	2·5 2·1	21 20	65 ± 5‡
5	UV21-W§	N-free + 0·02 N-NH_4 tartrate	1·4	103	100

* Half-neutralized with NaOH.
† Duplicate cultures.
‡ Estimates of nuclear ratio based on combined data from duplicate cultures.
§ Wild type derived by back-mutation of *am*.

Production of other enzymes by the mutant

The standard mutant strain and the derived back-mutant strain UV21–W, which should be virtually isogenic except for the locus under study, were compared for their production of other enzymes related in substrate specificity to glutamic dehydrogenase. The enzymes tested for were: isocitric acid and glucose-6-phosphate dehydrogenases (both specific for TPN); transaminases catalysing transfer of amino groups from L-alanine, L-aspartic acid, L-ornithine, L-phenylalanine and L-valine respectively to α-ketoglutarate. Evidence to be published later indicates that at least 4 different enzymes are involved in these five transaminase reactions. The two strains were grown for 48 hr. on N-free medium supplemented with 0·01 M-glutamic acid (half-neutralized with NaOH) and 0·01 N-ammonium tartrate.

For the assay of the two dehydrogenases extracts were prepared as for the assay of glutamic dehydrogenase, and the enzyme activities were determined by measuring the increase in rate of reduction of TPN in a spectrophotometer cell following addition of substrate. Isocitric dehydrogenase activity was increased nearly threefold by manganese ions. The results are given in Table 3. It will be seen that the levels of these two enzymes are similar in mutant and wild-type.

Table 3. *Glucose-6-phosphate and isocitric dehydrogenase activities in wild type and mutant*

	Activity (ΔE_{340}/min./mg. protein × 10^3)†	
Assay system	Mutant (*am*)	Wild-type (UV21-W)
1 ml. extract, 1 ml. TPN in buffer;* 0·2 ml. 0·1 M-hexose-6-phosphate added after measuring initial rate of reduction	270	230
After complete reduction of TPN by above system, 5 µmole α-ketoglutarate and 10 µmole NH_4Cl added, initial rate of reoxidation taken as measure of glutamic dehydrogenase	0	*c.* 90
1 ml. extract, 0·25 ml. 0·0067 M-$MnCl_2$, 1 ml. TPN in buffer;* 0·1 ml. 0·1 M-DL-isocitrate added after measuring initial rate of reduction	120	110

* 0·05 % TPN (31 % pure) in phosphate buffer.

† Activities corrected for slow TPN reduction in the absence of added substrate.

Extracts for assay of transaminases were prepared by grinding washed and blotted pads of mycelium with powdered glass and 2·5 vol. of pre-chilled phosphate buffer in an ice-cooled mortar for 15 min. followed by centrifugation at 2700 ***g*** for 5 min. The supernatants were dialysed against three changes of buffer at 0–5° for 3 hr. The transaminase activities were almost identical in the two strains compared on the basis of wet weight of mycelium, but on the basis of protein content the activities were all rather less in the mutant (Table 4). The mutant extract was much more turbid than the wild-type extract, and

Table 4. *Transaminase activities in wild type and mutant*

In each digest: 0·3 ml. extract in phosphate buffer, 15 µmole Na α-ketoglutarate, 15 µmole L-amino acid or water as control in a total vol. of 0·6 ml. Glutamate formation in water controls subtracted. Incubation at 34° for 15 min. for alanine and aspartate digests and for 60 min. for the others.

	Wild type	Mutant
Wet wt. mycelium (g.)	2·0	2·1
Extracted in (ml.)	5·0	5·2

	µmole glutamate formed			
Amino acid	Total	Per mg. protein	Total	Per mg. protein
Alanine	3·2	0·74	3·6	0·51
Aspartic acid (Na salt)	1·4	0·32	1·5	0·21
Ornithine	1·6	0·37	1·8	0·26
Valine	1·2	0·28	1·1	0·16
Phenylalanine	0·8	0·18	0·7	0·10

it may be that a higher proportion of protein of the mutant extract was associated with particles, the transaminases being in the soluble fraction (Fincham, 1951*b*). Other experiments in which glutamate formation was estimated semi-quantitatively by visual inspection of chromatograms (Fincham, 1953) revealed no consistent differences between mutant and wild-type in respect of transaminase activities.

DISCUSSION

The conclusion to be drawn from these experiments is that, in this case, the mutation of a single gene prevents the formation of a single enzyme. The experiments on enzyme production by heterocaryons make it seem likely that the mutation acts by removing part of the enzyme-producing system rather than by inhibiting or diverting the activity of a system with which the gene in question is not normally directly involved, since there is no clear indication of any suppression by mutant nuclei of enzyme production in the heterocaryons. Although it is not completely recessive in heterocaryons, the mutant allele, in Muller's (1932) terminology, seems to be an *amorph* rather than an *antimorph*. This conclusion would be strengthened if wild-type and mutant alleles could be obtained in the same nucleus.

The mutation seems to be specific in its effect, affecting only one enzyme activity out of eight examined. Isocitric dehydrogenase, glutamic dehydrogenase and the transaminases probably normally act in sequence in producing, aminating and regenerating α-ketoglutaric acid; the mutation has the effect of removing one step in the sequence without affecting the others.

It would be of great interest to know whether there is only one locus at which mutation has this effect. There is little information on this other than the fact that only two such mutations have been discovered among several thousand biochemical mutants and these are almost certainly allelic (J. A. Pateman, unpublished). A rather complicated situation has been uncovered by Yanofsky (1953) in the possibly parallel case of mutational elimination of tryptophan desmolase. Yanofsky obtained twenty-two mutants which were all alike in lacking tryptophan desmolase, and he also found three 'suppressor' mutations at different loci which restored the enzyme when combined with some of the mutants but not with others, and which differed among themselves in the mutants which they could suppress. The meaning of these observations is not clear, but if all twenty-two mutations are allelic a rather specific relation between a single locus and tryptophan desmolase production would seem to be indicated. The fact that some of the mutants were 'suppressable' may be taken to imply that they represent some alteration of the gene short of irreparable inactivation so that some (though never completely normal) activity can be restored by alteration of conditions in the cell as, for instance, by gene mutation of various kinds. It will be of considerable interest to see whether a similar situation exists in the present case.

Little can be said regarding the role of the gene in enzyme production. To speak of gene 'control' of enzyme synthesis may be misleading unless it is realized that the most that can be shown by present methods is that the wild type gene is an essential *component* of the enzyme-producing mechanism. Other components, including perhaps other genes or even cytoplasmic 'self-duplicating' units (Ephrussi, 1953), are by no means excluded. In the present case the concentration of enzyme is dependent, most strikingly, on the genotype, but also to some extent on conditions of growth. This may suggest that some kind of gene product, the concentration of which may vary, rather than the gene itself is directly involved in enzyme synthesis.

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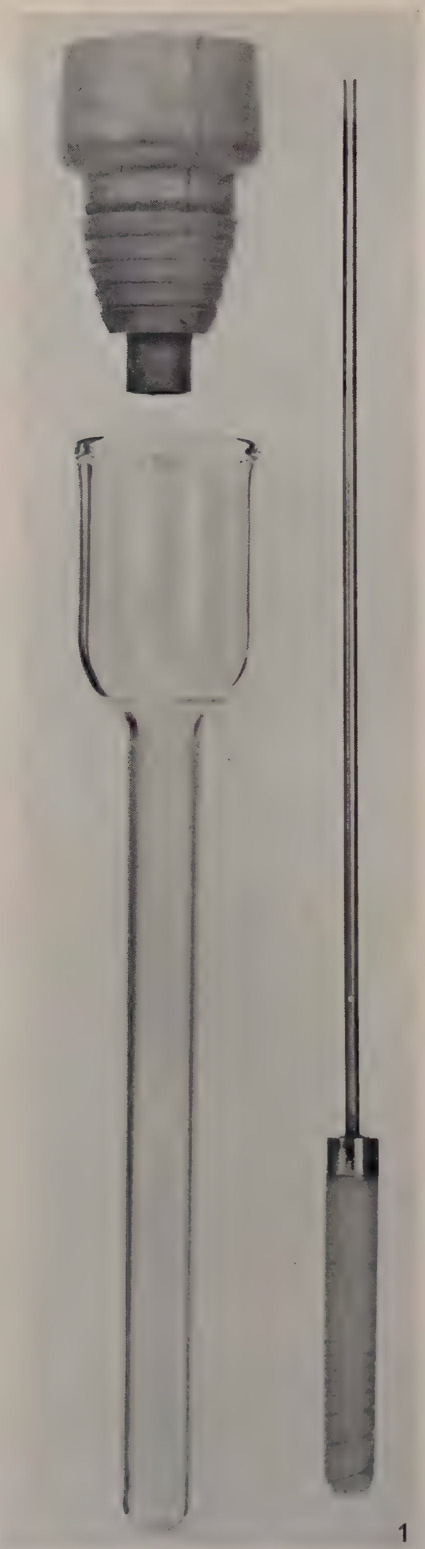

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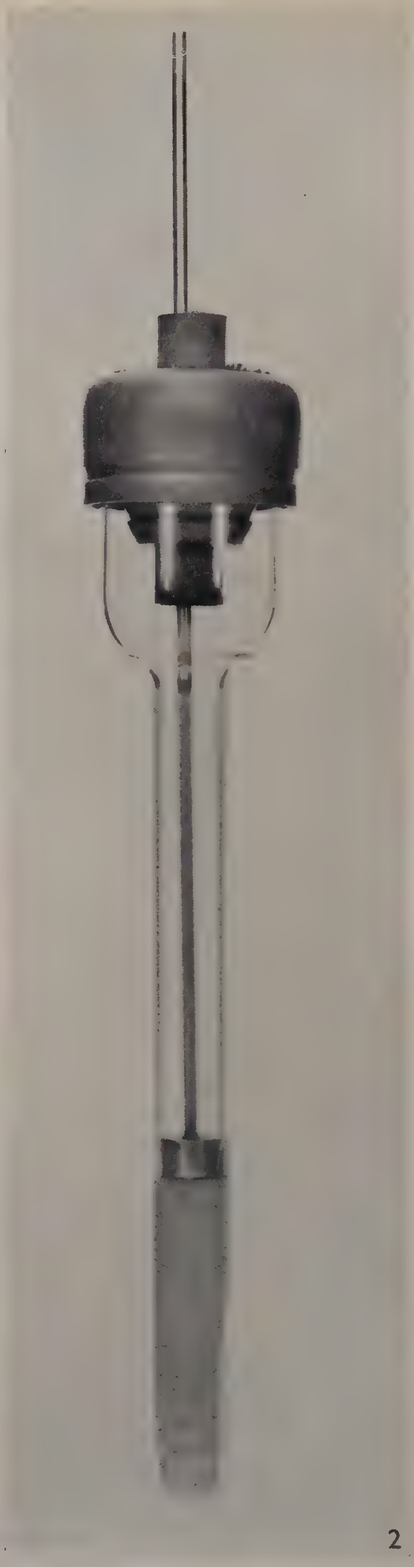

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G. R. F. Hilson & S. D. Elek—Nylon tissue grinder. Plate 1

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A Nylon Tissue Grinder

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SUMMARY: A tissue grinder is described, consisting of a nylon pestle mounted on a stainless steel shaft and revolving within a Pyrex tube; a screw is cut in the pestle to increase its efficiency. The whole device is sealed with a Subaseal bung and may be sterilized by autoclaving. Optimum grinding with minimum heating is produced by rotating the pestle at 500 r.p.m. with a small electric motor.

Tissue homogenizers are frequently required for biological and chemical studies. The usual type consists of a glass pestle fitting closely into a large glass test tube. In 1922 Hagan devised a simple form of grinder of this kind from two test tubes; this design was modified by TenBroeck (1931). Glaser & Coria (1935) elongated the pestle so that it could be attached to a motor, and Potter & Elvehjem (1936) fused glass beads on to the pestle, in order to tear up the tissue more effectively. This device is rotated by a cone-drive stirring motor at a speed of 80–90 r.p.m. (Parker, 1950) and is protected from contamination by a canopy of fibreglass cloth. Potter (1948) described an improved design and suggested a speed of about 1000 r.p.m. as optimal. Pyrex tubes with motor-driven pestles are, however, unsatisfactory in practice since the grinding surfaces wear away after being used 10–20 times, and the clearance is greatly increased. This leads to inefficient grinding, and large particles may be left; serious errors in enzyme determinations may arise in this way. For this reason Brendler (1951) suggested the use of pestles made of Lucite as it has better wearing qualities and powdered glass does not appear in the homogenates. Pestles made of stainless steel and Teflon (Pierce, Dubos & Schaefer, 1953) have also been used. The latter material is a synthetic tetrafluoroethylene resin resistant to organic solvents, acids and alkalies and to autoclaving. Nylon has similar qualities and was therefore used in the grinder described below. Mechanical rotation is far more efficient and convenient than grinding by hand, but the risk of overheating is present and must be guarded against. Ideally the design should combine high grinding efficiency with minimal heat production.

The type of grinder we used is shown in Pl. 1, figs. 1 and 2; it consists of a stout Pyrex test tube about 18 cm. long with an internal diameter of about 1 cm. Its upper part is expanded to receive a rubber 'Subaseal' bung with a central sleeve; in the illustration the sleeve is shown very much wider than the central spindle. For materials which might give rise to a dangerous aerosol, a bung should be fitted whose central sleeve is an easy fit on the spindle, tight enough to prevent the escape of fine particles of spray, but allowing rotation of the spindle. The spindle is a flexible stainless steel rod of about 3 mm.

diameter. This is set into the pestle, being locked in position by a small transverse screw. The pestle is turned on a lathe from a length of nylon rod 7·5 by 1 cm. to fit the Pyrex tube with a tolerance of about 0·1 mm. An Archimedean screw is cut into the lower third of the pestle, starting deeply and with a coarse pitch at the tip, and continuing upwards with diminution of both depth and pitch. The screw is cut in a direction opposite to the direction of rotation. The upper two-thirds of the pestle are smooth. All parts of the grinder may be autoclaved.

The grinder is used by placing in the tube about 5 ml. of the coarse suspension to be ground; the spindle of the pestle is attached to the chuck of a motor by a flexible drive. After starting the motor the pestle is firmly pressed into the suspension, the particles of suspension being forced up the channel of the screw. The particles are minced as the groove in which they travel becomes more and more shallow and they are finally ground between the smooth surface of the upper end of the pestle and the glass wall. The flexibility of the spindle produces an eccentric rotation of the pestle, resulting in a grinding force which is shearing in nature. When the pestle has reached the bottom, it is lifted up, with or without stopping the motor, and the process repeated.

Preliminary tests of the grinder, using bacterial suspensions, showed markedly decreased viable counts after grinding. It was not clear whether this was due to frictional heat or to pressure effects: high pressure is known to be lethal to bacteria, 5000 kg./cm.^{2} being said to be sufficient to sterilize cultures of *Salmonella paratyphi*-C (Vignais, Barbu, Macheboeuf & Bassett, 1952). We did not consider it practicable to investigate pressure conditions within the grinder, but were able to make estimations of temperature rise at the frictional surfaces. A copper-constantan thermocouple of fine wire was wound round the smooth part of the pestle, and while the latter was held stationary, the glass tube was rotated at varying speeds. This allowed continuous readings to be taken. Five ml. portions of a coarse suspension (*c.* 50 %, v/v) of rabbit liver were ground for up to 5 min. At high speeds much frictional heat was generated and such speeds are unsuitable. At 500 r.p.m. the temperature rise at the surface of a smooth pestle was not more than 3·5° in 5 min. and was less with the screw-cut pestles. The grinding efficiency of plain and screw-cut pestles at 500 r.p.m. was compared by making haematocrit readings of the same material ground for the same periods of time. Judged in this way, the Archimedean screw cut pestle appeared to be more efficient.

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Some Observations on Linkage Effects in Genetic Recombination in *Escherichia coli* K-12

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SUMMARY: Further recombination data have been obtained from matings involving different F$^+$ auxotrophic mutants of *Escherichia coli* strain K-12 with the same K-12 F$^-$ auxotroph. Variations in the distribution of sugar fermentation markers among the progeny have been explained by the influence of the 'contra-selected' F$^+$ auxotrophic marker. It is suggested that the data supports the conception of a single linear chromosome in K-12, which fragments randomly during recombination.

Genetic investigation of inhibition of the K-12 strain of *Escherichia coli* by norleucine (Clowes & Rowley, 1954) showed that the segregation of six sugar markers among progeny from the matings of the same ($T^-L^-B_1^-$) F$^-$ strain with two different ($T^+L^+B_1^+$) F$^+$ strains was comparable. This suggested that the same factors T$^+$, L$^+$, and B_1^+ were transferred from the F$^+$ to the F$^-$ parent in each case, accompanied by transfer of some unselected markers carried by the F$^+$ strain, in proportions depending only upon their proximity to the loci T$^+$, L$^+$ and B_1^+. This interpretation, based on the one-way transfer of genetic material proposed by Hayes (1952), suggests that the essential and uncomplicated function of the F$^+$ parent is to contribute that fragment of the bacterial chromosome (or chromosomes) needed by the F$^-$ parent to provide the complete genotype demanded by the selecting medium. However, further matings involving the same F$^-$ strain showed differences in the distributions of these sugar markers among the progeny when compared with similar data from the previous matings. That these differences are due to complications introduced by the relative positions of the selected markers of the F$^+$ parent with respect to those of the F$^-$ parent and to these sugar markers is shown in the following.

MATERIALS AND METHODS

Strains of Escherichia coli *used*

(*a*) Wild type Strain K-12. This strain grows on a minimal medium (Davis & Mingioli, 1950) unsupplemented by amino acids or vitamins. It is sensitive to streptomycin and ferments maltose, lactose, D-galactose, D-xylose, D-mannitol and L-arabinose (Mal$^+$, Lac$^+$, Gal$^+$, Xyl$^+$, Mtl$^+$, Arab$^+$). This strain crosses with F$^-$ strains and is thus F$^+$.

(*b*) *Strain* 58–161. A methionine requiring mutant (M$^-$) of K-12, having the same fermentation pattern (Mal$^-$, Lac$^+$, Gal$^+$, Xyl$^+$, Mtl$^+$, Arab$^+$) as the parent strain. Both F$^+$ and F$^-$ types were used.

(*c*) *Strain* W-677. A threonine, leucine and thiamine (T$^-$, L$^-$, B_1^-) requiring auxotroph of K-12. This strain was derived from strain W-1 (T$^-$, L$^-$, B_1^-,

Mal_1^-, Lac_1^-) by incorporating the inability to ferment galactose, xylose, mannitol and arabinose in a series of separate steps, the Gal^- locus being designated Gal_5^- (E. M. Lederberg, personal communication). F^+ and F^- forms of this mutant were used together with the streptomycin-resistant mutant (St^R) of the F^- form (W-677/St^R/F^-). The genetic markers carried by W-677 are allelic with those of strain W-945, which has been the basis of other genetic investigations (Cavalli, Lederberg & Lederberg, 1953), for the loci T, L, B_1, Mal and Lac since both strains are derived from W-1. The St and Xyl loci have been assumed to be allelic and the allelism of the Xyl loci is supported by recombination data (L. L. Cavalli-Sforza, personal communication).

(*d*) The other auxotrophic strains of K-12 (designated 'J' strains) used in this investigation are listed in Table 1.

Table 1. *Derivation and requirements of 'J' strains of* Escherichia coli

Strain:	J4–5*	J5–3*	J5–10*	J6–2*	J13–4
Derivation:	Double mutant by irradiation	From the same proline-requiring mutant		From J4–5	From 58–161
Requirements:†	Tryptophan, Histidine (Tr^-, H^-)	Proline, Methionine (P^-, M_2^-)	Proline, Histidine (P^-, H^-)	Proline, Histidine, Tryptophan (P^-, H^-, Tr^-)	Methionine, Isoleucine or α-aminobutyric acid (M_1^-, iL^- or α–aB^-)

* Derived from K-12 passing through a Lac^- mutation (B. D. Davis—personal communication).

† All growth factors required in minimal concentrations of 20 μg./ml.

All five 'J' strains are able to ferment maltose, D-galactose, D-xylose, D-mannitol and L-arabinose. In addition, J5–3, J5–10 and J13–4 ferment lactose but J4–5 and J6–2 are unable to ferment it. All strains produced recombinants with W-677/F^- and were inferred to be F^+. The presence of prototrophic recombinants from the mating of J5–3 (P^-M^-) with 58–161 (M^-) on MM was taken to indicate that the methionine markers carried by these strains are non-allelic. For convenience, the M^- locus of 58–161 will be designated M_1^-, and that of J5–3, M_2^-.

Media and recombination techniques were the same as described previously (Clowes & Rowley, 1954).

Biochemical tests. Master plates of purified recombinant colonies, prepared as described previously (Clowes & Rowley, 1954), were used for the testing of fermentation reactions and auxotrophic requirements by the replica plating method of Lederberg & Lederberg (1952), all sugar fermentation tests being carried out on eosin-methylene blue medium.

RESULTS

Table 2 shows the segregation of the six sugar markers Mal_1, Lac_1, Gal_5, Xyl, Mtl and Arab among the recombinants resulting from the matings of W-677/F^- (or W-677/St^R/F^-) with K-12, 58–161/F^+ and the five 'J' strains on minimal

Table 2. *Segregation of sugar fermentation markers in progeny of matings of various* F^+ *strains with strain* W-677/St^R/F^- or W-677/F^-

F^+ strains are Mal^+ Gal^+ Xyl^+ Mtl^+ $Arab^+$. W-677 is T^- L^- B_1^- Mal^- Lac^- Gal^- Xyl^- Mtl^- $Arab^-$.

Group	F^+ parent	F^+ markers	Selecting medium	Colonies prototrophic (%)	No. colonies examined	Recombinants fermenting (%)					
						Mal	Lac	Gal	Xyl	Mtl	Arab
A	K-12	St^s	MM + St		226	4	26	1	13	12	0
			MM + St + B_1	13	183*	1	35	0	3	2	3
	58–161	M_1^-	MM		240	6	27	4	12	12	5
			MM + B_1	11	204*	1	37	1	1	1	2
B	J4–5	H^-, Tr^-, Lac^-	MM		351	23	—	8	27	26	10
			MM + B_1	27	142*	5	—	4	5	5	4
	J5–10	P^-, H^-	MM		271	24	3	9	26	31	18
			MM + B_1	25	223*	8	3	4	7	8	3
	J6–2	P^-, H^-, Tr^-, Lac^-	MM		122	20	—	7	20	22	9
			MM + B_1	27	73*	3	—	0	2	2	0
C	J5–8	P^-, M_2^-	MM		410	12	5	6	10	11	8
			MM + B_1	16	173*	3	2	2	1	2	2
D	J13–4	M_1^-, iL^- or α-aB^-	MM		376	8	33	9	5	4	8
			MM + B_1	0	100*	1	56	11	3	3	8

MM = minimal medium; B_1 = thiamine supplement; St = streptomycin supplement.

* Fermentation figures quoted for B_1^- recombinants only

Table 3. *Effect of supplementing recombination plates with* F^+ *growth factors on segregation of sugar markers among progeny from matings of various* F^+ *strains with strain* W-677/F^- or W-677/St^R/F^-

F^+ parent	Selecting medium	'Contra selected' markers	Ratio no. recombinants to those on MM	Prototrophic recombinants (%)	No. of recombinants examined	Class of recombinant	Recombinants fermenting (%)					
							Mal	Lac	Gal	Xyl	Mtl	Arab
J4–5	MM+H	Tr	1·2	100	96	Pr	24	—	7	30	32	14
	MM+Tr	H	1·0	100	97	Pr	28	—	3	33	34	10
J5–3	MM+P	M_2	1·3	70	35	P^-	14	75	10	28	24	14
	MM+M	P	3·1	35	160	M^-	31	3	10	34	39	15
J5–10	MM+P	H	1·5	66	65	P^-	11	60	15	31	32	15
	MM+H	P	0·8	100	121	Pr	27	6	17	30	30	16
J6–2	MM+P	H, Tr	2·1	70	37	P^-	14	—	8	16	16	5
	MM+H	P, Tr	1·1	100	85	Pr	22	—	13	26	23	12
	MM+P+H	Tr	2·0	80	41	P^-	17	—	10	20	20	7
	MM+St+P+H+T_r	St	0·8	63	80	Pr	4	—	1	10	17	1
J13–4	MM+M	iL (or α-aB)	2·5	50	167	M^-	11	40	4	5	4	8
	MM+iL	M_1	1·5	55	61	iL^-	17	34	11	23	28	8
	MM+St	St, M_1, iL (or α-aB)	0·15	100	49	Pr	6	30	0	8	6	0
	MM+St+M+iL	St	0·2	65	50	Pr	6	21	3	9	6	3
	MM+α-aB	M_1	0·7	100	50	Pr	12	26	8	2	0	8

Pr denotes prototrophic recombinants.

MM = minimal medium. Supplements: H = histidine; Tr = tryptophan; P = proline; M = methionine; St = streptomycin; iL = isoleucine; α-aB = α-aminobutyric acid.

medium (MM) and minimal medium supplemented with vitamin B_1 ($MM+B_1$). These matings may be conveniently separated into four groups of similar segregation patterns. It is apparent from these different groups that the genotype of the F^+ parent has a significant effect on the genotype of the recombinants, under the experimental conditions used. In order to investigate this effect more fully, certain matings were repeated and seeded on minimal media supplemented with the individual growth factors of the F^+ parent, in cases where this parent has more than one growth requirement. The results of such matings are summarized in Table 3. The effect of the addition of the F^+ growth factors shows that the supplements methionine, isoleucine and proline affect the recombination rate, the growth requirements of the recombinants and their sugar fermentation pattern. All recombinants arising on MM supplemented with either histidine or tryptophan were capable of growth on unsupplemented MM and their sugar fermentation patterns were similar to that arising on MM alone (Table 2).

Table 4. *Segregation of sugar markers from the mating* W-677/$F^+ \times$ 58–161/F^-

Selecting medium	Ratio recombination rate to that on MM	Recombinants growing on MM (%)	No. colonies tested for sugar fermentation	Progeny *not* fermenting (%)					
				Mal	Lac	Gal	Xyl	Mtl	Arab
MM	1	100	412	21	4	8	26	25	7
$MM+B_1$	1·9	55	130*	23	5	4	40	34	4

* = B_1^- recombinants; MM = minimal medium; B_1 = thiamine supplement.

DISCUSSION

One of the difficulties in studying the genetics of *Escherichia coli* K-12 has been to account for the position of the known markers on the basis of one or more straight or branched chromosomes. The present data are discussed on the basis of a multichromosome system and then on the basis of limited transfer of fragments of a single chromosome.

Multi-chromosome hypothesis. Watson & Hayes (1953) suggested that the present markers could be arranged in three linkage groups, characterized by the loci St-Mal_1, M_1-B_1 and TL-Lac_1, and Hayes (1953*b*) suggested that recombination data could be interpreted on the basis of at least three discrete chromosomes. In recombination a one-way transfer of usually only one of the chromosomes of the F^+ parent was postulated. If this transferred chromsome carries one selected marker only, then it follows from the above hypothesis that other loci carried by this chromosome should appear in the progeny to the extent of 50–100 %, depending upon whether there is no linkage (50 %) or complete linkage (100 %) of the unselected marker in question with the selected marker. Other markers on any chromosome not carrying a selected marker should appear in the progeny at a small random rate, depending only upon the frequency with which this chromosome happens to be transferred from the F^+ parent in addition to that chromosome carrying the selected markers from the F^+ strain.

In an example quoted by Watson & Hayes, the cross of W-677/$F^+ \times$ 58–161/F^- on MM+B_1 where only the chromosome carrying the M_1^+ locus is being selected, there was a transfer of 61 % B_1 and 21 % Mtl. The loci B_1 and M_1 are postulated to lie on the same chromosome, and Mtl on another, yet there does not appear to be any clear-cut difference between the so-called random transfer of Mtl and the linked transfer of B_1.

In a mating of J5–3 ($P^-M_2^-B_1^+T^+L^+$) and W-677/F^- ($P^+M_2^+B_1^-T^-L^-$) on MM+M+B_1 we found that of 82 recombinants examined 53 were $M_2^+B_1^-$, 22 were $M_2^-B_1^+$, 6 were $M_2^+B_1^+$ and 1 was $M_2^-B_1^-$. This shows that the M_2 locus is also linked to B_1 and by the Watson & Hayes hypothesis it is situated on the same chromosome as both M_1 and B_1.

A consideration of some of the present data shows that the proposed random transfer of some markers is considerably affected by the transfer of a supposedly non-homologous chromosome. For example, in the cross J5–3 × W-677/F^- on MM (Table 2) the chromosomes carrying the selected markers (B_1^+, T^+ and L^+) would need to be contributed by the F^+ parent to all viable recombinants. However, since the F^+ chromosome carrying B_1^+ also carries the M_2^- marker of the F^+ parent, crossover in this chromosome is necessary to secure a viable recombinant, which must carry the loci M_2^+ and B_1^+, i.e. the locus M_2^- is 'contra-selected'. On MM+M, the same chromosomes are transferred from the F^+ parent, but the class of progeny which carry the M_2^- locus is now viable. If this class is examined (M_2^- colonies, Table 3) the percentage transfer of the loci Mal, Xyl and Mtl is seen to differ significantly from that of the M_2^+ class of recombinants (see Table 2). In this mating, the transfer of 10–12 % of Mal, Mtl and Xyl on MM (Table 2) would be supposed random on Watson & Hayes's interpretation. However, by transferring an additional piece of another F^+ chromosome (that containing the M_2^- locus) the simultaneous transfer of these sugars is increased to 31–39 %. The inference must be that the transfer of these sugar markers is not random, and furthermore that in some way they are linked with M_2 and thus to B_1.

It is evident on consideration of the recombination data available (Lederberg, 1947; Lederberg, Lederberg, Zinder & Lively, 1951; Cavalli *et al.* 1953; Hayes, 1953*a*; Rothfels, 1952) that the known markers tend to lie in the linkage groups as postulated by Watson & Hayes (1953), but that these form discrete chromosomes is by no means certain as the present data show.

Limited transfer of fragments of a single chromosome. Previous work, as well as the above result, can be interpreted on the basis of a single linear chromosome which fragments at random during recombination, a possibility considered by Watson & Hayes (1953). The three groups of markers must then be considered to occupy small separated segments of this single chromosome. It is necessary to visualize fragmentation of this chromosome at some time during recombination since not all markers are transferred to an extent of 50–100 % even when the F polarity is reversed (e.g. in the case of Gal and Arab in the two crosses of 58–161 and W-677; Tables 2 and 4). The appearance of any unselected marker of the F^+ parent in the recombinants must depend on the frequency of its incorporation on the same chromosomal fragment as a selected marker from the F^+ parent, this transfer being proportional to the proximity of the two loci. This implies that any transfer, however small, is due to some degree of linkage.

On this concept of limited transfer, the experimental evidence may be interpreted as follows. In the cross J 5–3 ($P^-M_2^-$) × W-677/F^- on MM (Table 2) the loci T^+, L^+ and B_1^+ are selected from J 5–3 but the M_2^- locus is contra-selected and the transfer of sugar markers Mal, Xyl and Mtl is small (Mal 12 %, Xyl 10 %, Mtl 11 %). On MM + M however (Table 3), where the M_2^- locus can be carried by viable recombinants, an increased proportion of these latter progeny possess the sugar markers (Mal 31 %, Xyl 34 %, Mtl 39 %), the increased transfer being presumably due to their linkage with M_2. The similarity in the distribution of these sugar markers (Table 2) among progeny from matings on MM of group 'B', where all F^+ parents carry the M_2^+ allele, and those of J 5–3 on MM + M supports the explanation that the differences on MM are due to contra-selection of the M_2^- locus. The transfer of the markers Mal, Xyl and Mtl are of a similar order in the crosses both of 58–161/F^+ and J 5–3 with W-677/F^- on MM (Table 2), which confirms the proximity of the M_1 and M_2 loci. It seems likely that contra-selection for either M_1^- or M_2^- is the reason for the lower transfer in both these crosses compared with the analogous results within group 'B'.

That strain K-12 also resembles strain 58–161 in this respect could be attributed to the contra-selection of the St^s locus which has also been shown to be linked to Mal, Xyl and Mtl by previous workers (Cavalli *et al.* 1953). Further evidence to support this suggestion can be got from consideration of the cross of J 6–2 × W-677/St^R/F^- (Table 3). On MM the progeny from this mating resembles that of other group 'B' strains, but when contra-selection for the St^s locus is applied, by selecting on MM + St + P + H + Tr, the transfer of markers is reduced, and is similar to that observed in the cross involving K-12 on MM + St.

The differences in Lac^+ transfer between crosses involving either J 5–3 or J 5–10 and those in which either 58–161 or K-12 is the F^+ parent may also be explained by the effects of contra-selection, in this case of the P^- locus. In the mating of J 5–10 with W-677/F^- on MM × P (Table 3), there is a larger transfer of Lac^+ among the P^- recombinants (60 %) than among the P^+ recombinants (6 %). This suggests P is closely linked with Lac and that when P^- is contra-selected, as in J 5–3 or J 5–10 × W-677/F^- on MM, a reduced transfer of Lac^+ to the recombinants results.

The matings of J 13–4 (iL^- or α-aB^-, M_1^-) are more complex and not entirely explicable. The main distinctions in the progeny from this strain are the very small transfer of the markers Xyl and Mtl on MM which is less than from strains of group A, and the lack of variation in the transfer of these markers on supplementing with M. This would be explained by a close linkage of iL to Xyl and Mtl and the location of this marker between M_1 and the Xyl-Mtl pair. In several recombinations selected on MM + iL, no iL^- progeny could be detected. However, in other recombinations iL^- recombinants were detected but the clones were always much smaller than those of any other class of recombinants.

The distribution of sugar markers among these iL^- recombinants shown in Table 3 shows an increase of transfer of the markers Mal, Mtl and Xyl in proportions similar to that among recombinants where the M_1^- locus is not contra-selected, which would be expected were the iL locus situated between the M_1 locus and the loci Mal, Xyl and Mtl. An attempt was made to investigate the linkage of M_1 and iL by seeding the cross J13–4 × W-677/St^R/F^- on MM + St + iL + M. On this medium all recombinants fell into the two classes $St^RiL^+M_1^+$ and $St^RiL^+M_1^-$ and this lack of iL^- recombinants was confirmed by the fact that recombinants arising on MM + St + iL were all prototrophic. It was thought that this was due to the closer linkage of St and iL which would prevent their separation. No effects on recombination rate, growth requirements and sugar patterns of the progeny of the cross J13–4 × W-677/F^- were found by supplementing with α-aminobutyric acid. However, the alternative growth requirement of isoleucine or α-aminobutyric acid of this auxotroph makes interpretation of its mating difficult.

It should be emphasized therefore that the division of the F^+ strains used in this study into the four groups in Table 2 does not imply any fundamental difference in the mechanism of genetic transfer among these strains. The differences in the distribution of the various markers among their recombinants probably arise as a result of contra-selection of F^+ markers imposed by the selecting medium. If all contra-selection were removed it is likely that the transfer of unselected markers would be comparable in all crosses and would reflect only the various linkages of these markers to T^+, L^+ and B_1^+, the common selecting markers in all crosses.

Construction of a linkage map

On the basis of limited transfer, the data presented and that of other workers may be interpreted to construct a linkage map of the K-12 chromosome as follows.

In all the matings involving W-677/F^- on B_1 media (Table 2), the B_1^+ recombinants form *c.* 20 % of the progeny. We may thus infer that (TL) and B_1 are linked. Of the other quoted markers, selection for TL brings over only a significant amount of Lac, as shown by the crosses of group 'A' on B_1 media, and supports the findings of Watson & Hayes (1953). The introduction of selection for B_1 on MM reduces the transfer of Lac from *c.* 36 to *c.* 26 %. This reduction appears to be significant ($\chi^2 = 7{\cdot}3$) and suggests that Lac_1 and B_1 lie on opposite sides of TL.

The large reduction in the transfer of Xyl and Mtl in all crosses on eliminating selection for B_1 (Table 2) implies the linkage of these sugar markers with B_1 and not with TL, although the relative positions of the two sugar markers cannot be fixed unequivocally, and suggests the order

$$(\text{Mtl-Xyl}) - B_1 - \text{TL} - \text{Lac}_1.$$

That M_1 and B_1 are linked has been shown by many other workers (Hayes, 1953*a*; Cavalli *et al.* 1953; Watson & Hayes, 1953) and this is supported by the appearance of *c.* 50 % of the one marker when the other is selected (Tables 3 and 4). The relative positions of M_1 and B_1 is suggested by consideration of the transfer of Xyl and Mtl when either M_1 or B_1 is selected. If we suppose M_1 to be situated to the right of B_1; in the cross W-677/F^+ × 58–161/F^- we would expect on MM where the M_1 locus only is selected that there would be little transfer of Xyl and Mtl but that on B_1 medium a larger transfer would result. If M_1 were situated between B_1 and Mtl + Xyl, however, the reverse effects would be expected, and the transfer on MM should be little affected on supplementing with B_1. The data (Table 4) show transfers of 26 % Xyl, 25 % Mtl on MM and 40 % Xyl, 34 % Mtl on MM + B_1. The figures for Mtl do not indicate any significant increase on supplementing with B_1 ($\chi^2 = 3{\cdot}85$) but those of Xyl suggest that the variation is not random ($\chi^2 = 8{\cdot}5$). These results are difficult to reconcile with either alternative. However, the fact that the transfer of only one of these closely linked markers seems to be increased on selecting for B_1, and that when M only is transferred there is still a large amount of transfer of both markers throws doubt on the location of B_1 to the right of M. Similarly, in the cross J 5–3 × W-677/F^-, the situation of M_2 to the right of B_1 would allow a significant transfer of these sugar markers on MM and the transfer should be little affected on supplementing with M. If M_2 were to the left of B_1, the transfer on MM should be small and greatly increased on MM + M. The results shown in Tables 2 and 3 suggest that selection for M_2 has a significant effect on the transfer of either Xyl ($\chi^2 = 36$) or Mtl ($\chi^2 = 44$). The location of M_2 to the left of B_1 would appear the only satisfactory arrangement. It should be pointed out however that as well as the discrepancy for Xyl transfer in the first mating noted above, the fact that there is a residual transfer of 10 % Xyl and 11 % Mtl on selection for B_1 and contra-selection for M_1 or M_2 would require an unusually high degree of double crossover. The unequivocal location of M_2 to the left of B_1, and the fact that contra-selection of either of the M_2 or M_1 loci in the presence of selection for B_1 as in the crosses of either J 5–3 and 58–161 with W-677/F^- on MM (Table 2) gives similar transfers of Xyl and Mtl suggest that the most probable location of both M_1 and M_2 are to the left of B_1.

Tables 3 and 4 show that when M_1 is selected or when B_1 is selected with no contra-selection for M_2, a small transfer of both Gal and Arab is effected. This suggests that these fermentation loci which have been shown to be closely linked with the locus for resistance to norleucine inhibition, Nl (Clowes & Rowley, 1954) are linked to M_1 and M_2 rather than B_1 or TL and implies the arrangement:

$$(\mathrm{Nl}\text{-}\mathrm{Gal}_5\text{-}\mathrm{Arab})\text{–}(\mathrm{Xyl}\text{-}\mathrm{Mtl})\text{–}(\mathrm{M}_1\text{-}\mathrm{M}_2\text{-})\text{–}\mathrm{B}_1\text{–}\mathrm{TL}\text{–}\mathrm{Lac}_1.$$

The Mal_1 locus is seen to be linked to M_1 from Table 4. Its percentage transfer and the results of Cavalli *et al.* (1953) suggest it is linked to B_1 less than Xyl. However, in matings where B_1 is transferred, the application of contra-selection for M_2 would be expected to reduce to the greatest amount those markers nearest to M_2. The results, shown in Table 3 on contra-selecting for M_2 in the cross of J 5–3, give a reduction in transfer of Mal 31 to 12 %, Xyl 34 to 10 % and Mtl 39 to 11 % and do not seem to suggest any difference in the degree of linkage of either Mal_1, Xyl or Mtl with M_2. The relative position of Mal_1 with respect to Xyl and Mtl cannot therefore be postulated exactly. The linkage St-Mal_1 has been shown by many workers. The decrease in transfer of St in an analogous manner to that of Mal, Xyl and Mtl when M_1 is contra-selected has been noted in a previous paper (Clowes & Rowley, 1954) where in the cross J 5–10/St^R × W-677/F^- on MM, 27 % of the recombinants were St^R as compared with a transfer of 10 % in the parallel 58–161/St^R/F^+ cross. The linkage between St and Mal and Xyl, quoted by Cavalli *et al.* (1953) would suggest the inclusion of these markers in the order:

$$(\text{Nl-Gal}_5\text{-Arab})\text{–}(\text{St-Mal}_1)\text{–}(\text{Xyl-Mtl})\text{–}(\text{M}_1\text{-M}_2)\text{-B}_1\text{–TL–Lac}_1.$$

In the cross of K-12 × W-677/St^R/F^- on MM + St, the similar degree of transfer of Mal, Xyl and Mtl would be explicable by the linkage of St to these markers to the same extent as M_1, together with contra-selection of the St^s locus. This is substantiated in the cross of J 6–2 (Table 3) where contra-selection for the St^s locus causes a reduction in transfer of these sugar markers to values similar to the K-12 cross.

The removal of contra-selection for proline in the crosses of J 5–10 (Table 3) allows a greater transfer of Lac, from which linkage of these two loci is inferred; this would explain the small degree of transfer of Lac in crosses involving P^-F^+ strains on MM. The data are compatible with the location of P between TL and Lac_1, since imposing contra-selection for P on the selection for TL reduces the transfer of Lac from *c.* 24 to 3 %.

The results of the crosses involving J 13–4 can be interpreted if the locus iL is between the M_1-M_2 and the Xyl-Mtl markers. However, in view of the alternative growth requirement of this auxotroph (M-iL or α-aB), this result cannot be more than tentative.

From the above considerations, it seems likely that the investigated markers lie in the order:

$$(\text{Nl-Gal}_5\text{-Arab})\text{–}(\text{St-Mal}_1)\text{–}(\text{Xyl-Mtl})\text{–iL-}(\text{M}_1\text{-M}_2)\text{-B}_1\text{–TL–P–Lac}_1.$$

The brackets imply uncertainty as to the relative positions of the markers enclosed.

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Autoadaptation of Bacterial Viruses and its Effect on Bacterial Variation and Evolution

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SUMMARY: The adaptation of viruses to attack new hosts which originally are resistant greatly widens the sphere of influence of viruses on bacterial variation and evolution. The viruses attacking diphtheria bacilli are highly specific in their host range activity but many of them are readily adapted to lyse strains of other serological types. The range of adaptability of viruses is largely unknown and studies on the specificity and adaptability of bacterial viruses are complicated by the phenomenon of autoadaptability. Although virus-carrying bacterial strains are normally resistant to the viruses with which they are symbiotically infected it is now found that in many cases the viruses can be readily adapted to attack and lyse the parent bacterial cells. This has been found to occur spontaneously in cultures, and accounts for many cases of bacterial variation and dissociation. The existence of closed evolutionary chains in lysogenic bacterial strains provides the explanation of many phenomena and difficulties in studies of phage typing and specificity. A further effect of significance in this field is the possibility of obtaining hybrid viruses by genetic recombination between an externally infecting virus and a virus already symbiotically infecting a bacterial cell. These effects can be elicited experimentally but they have also been observed to occur spontaneously in stock cultures and, it is suggested, may account for processes of bacterial variation and evolution controlled by viruses.

It has been shown that bacterial viruses may constitute an important factor in controlling bacterial evolution (Hewitt, 1952, 1953) supplementing the normal biological processes of natural selection and the survival of the fittest. Bacterial viruses are of very wide occurrence, and among many bacterial species a high proportion of strains are symbiotically infected with viruses, but these are frequently highly specific in their range of activity. Despite the common occurrence of bacterial viruses therefore the chance of any bacterial strain encountering a specifically active virus would be remote were it not for the phenomenon of adaptation by which viruses can be trained to attack originally resistant bacteria. It should be emphasized that the term adaptation is used in its widest sense without prejudice to the mechanism of the process which may well be by means of mutation and selection.

This communication is concerned with some of the factors involved in this adaptation process which are largely unknown but are of importance in practical problems of bacteriology and phage typing as well as in more speculative aspects.

MATERIALS AND METHODS

Most of the experiments have been conducted with diphtheria cultures, both recently isolated and strains in laboratory culture which have been described previously (Hewitt, 1947). Both tryptic digest and papain digest broths have been used for the cultivation of bacteria and propagation of viruses. It was found necessary to test each batch for suitability for virus propagation before use. For the detection of lysis on solid media the incorporation of 10 % horse serum as a growth adjuvant was found desirable. Special culture media for specific purposes are described in later sections.

In preliminary experiments the virus preparations used were the supernatant fluids from centrifuged cultures. Since diphtheria bacilli are readily killed by heating, sterilization of the preparations could be attained by heating at 50° for 30 min. The diphtheria phages are destroyed by heating at 55° and for obtaining sterile unheated virus preparations filtration was used. There are heavy losses of virus in Pasteur Chamberland L3 candles and Seitz filter pads but filtration through sterile collodion membranes of average pore diameter 0·7 μ was found satisfactory. General methods of virus propagation and testing have been described previously (Hewitt, 1952).

RESULTS

Detection of lysogenicity

In order to demonstrate that a bacterial strain is symbiotically infected with a virus it is necessary to have available a susceptible bacterial strain which will be lysed by any virus particles liberated by the bursting of an occasional lysogenic bacterial cell. Fisk (1942) placed drops of suspected lysogenic strains on smears of other bacterial strains on agar plates and examined for evidence of lysis. In the case of many diphtheria strains which yield rough irregular growth appearances on solid media traces of lysis are difficult to detect, and no clearing of broth cultures by viruses can be observed. It is often necessary therefore, to augment the potency of the virus preparation by attempting its propagation on the suspected sensitive strain before the possibility of lysogenicity can be excluded. In many lysogenic strains an occasional bacterial cell bursts, liberating virus particles, and the number of these can sometimes be increased by ultraviolet irradiation of the culture (Lwoff, Siminovitch & Kjeldegaard, 1950). It is probably impossible to be certain that a strain is not lysogenic, but conversely, proof that bacteria are carrying a symbiotic virus can be quite definite.

The procedure adopted for detecting bacterial lysogenicity is as follows: the culture or supernatant fluid is first spotted on to a number of possibly sensitive strains on agar plates, then a similar test is applied to strains first subjected to ultraviolet irradiation, and finally the supernatant fluids or sterile filtrates from ultraviolet irradiated cultures are first propagated in broth cultures of the sensitive strain before testing for lytic activity on agar plates.

In a preliminary experiment 24 diphtheria strains were each added to cultures of 6 other strains selected for possible sensitivity. The supernatant fluids from each mixed culture were added to fresh subcultures of the 6 possibly susceptible strains and this serial propagation was continued several times. When the supernatant fluids were spotted on agar plate cultures of the 6 strains it was found that in the case of 20 out of the 24 strains some lytic activity had been produced against strain H10. This strain was therefore selected as a suitable indicator strain for the detection of lysogenesis and for the propagation of diphtheria phages. Similarly, Smith & Jordan (1931) had selected an indicator diphtheria strain in their experiments which will be commented upon later.

Using H10 as propagating strain a considerable number of fairly potent virus preparations have been obtained. The specificity of the phage preparations was quite narrow; they produced lysis only in H10 and a few closely related strains. It was found possible, however, to adapt the virus preparations to lyse strains of other serological types by several serial passages with them. Different strains appeared to vary in the ease with which viruses could be adapted to lyse them, but having been adapted to lyse one strain the preparation could then be re-adapted to lyse fresh strains and so on apparently indefinitely. In some cases the original specificity was immediately lost; in other cases the host specificity was only slightly modified in each adaptation and the ability to attack the original host was lost only after several re-adaptations.

The range over which the host specificity of viruses can be altered is not known, so attempts were made to adapt various staphylococcus phages to lyse diphtheria strains. None of the staphylococcus phages examined had any direct lytic effect on diphtheria strains, so passage of the supernatant culture fluids in fresh subcultures of the diphtheria strains was carried out as already described in the case of diphtheria phages. After a varying number of serial passages, varying from 2 to 18, a number of supernatant fluids obtained from staphylococcus phages added to the diphtheria indicator strain were found to possess definite lytic action on H10. When other diphtheria strains were examined it was found that lytic filtrates could be obtained in some cases but in others no lysis was observed. This unexpected result of obtaining a lytic agent for diphtheria bacilli by serial subculture with staphylococcus filtrates was subjected to careful examination in order to provide a reasonable explanation. The most obvious explanation of accidental contamination was eliminated by frequent repetitions of the experiment under different conditions. The staphylococcus phages had not however been quantitatively converted into diphtheria phages as shown by titrations in successive passages. The amount of diphtheria phage appearing was quite independent of the fall in staphylococcus phage titre which occurred by dilution in successive passages. Also antisera prepared against the original staphylococcus phage had no measurable effect on the diphtheria phage preparations. Experiments with antisera against diphtheria phages were unsatisfactory since sera of reasonable potency could not be obtained. The phages were not of high enough titre to be good antigens, and possibly the antigenic response of the rabbits had been impaired since they had to be protected against the toxin present in the diphtheria filtrates either passively with antitoxin or by preliminary active immunization with diphtheria toxoid.

Since neither chance contamination nor quantitative conversion of staphylococcus phage to diphtheria phage could account for the results a more complicated explanation appears necessary. There may, for example, be an interaction between the staphylococcal filtrates and possible lysogenic phages carried by the sensitive diphtheria strains, as found for example by Anderson & Felix (1952) in the case of typhoid bacilli. More information was needed about the phage-host relationship in the case of *Corynebacterium diphtheriae*, about the adaptability of phages and about the possibility of genetic recombination.

Further experiments described in this and subsequent papers indicate that the diphtheria strains investigated are indeed lysogenic and capable of both adaptation and genetic recombination. In some cases the diphtheria strains were found to be carrying two or more distinct viruses and there seems little doubt that the development of lysins by subculturing staphylococcus filtrates with certain diphtheria strains is related to the presence of viruses carried by lysogenic strains.

Autoadaptation

In an experiment designed to lead to the isolation of a new series of viruses from 11 diphtheria strains, each one was subcultured with each of 10 strains making 110 cultures in all. After 18 hr. incubation the supernatant fluid from each of the mixed cultures was added to a fresh subculture of the propagating strain. The serial passages were continued several times, and then the supernatant fluids were tested for lytic activity on serum agar plates. In twenty-four cases some lysis was observed and the new viruses were isolated. Perhaps the most interesting observation, however, was that in three cases which had been included as controls the lytic activity observed was in cultures in which a strain had been subcultured with supernatant fluids obtained from previous serial subcultures of the same strain and no other bacterial strain had been added. It was found in fact that the symbiotic viruses carried by these strains had become adapted to lyse their own parent-carrying strains. Normally, of course, the host bacterium is resistant to lysis by its symbiotic virus, otherwise the host could not survive. This unexpected possibility of obtaining readily from a lysogenic strain a virus lysing the parent bacterium opens up new potentialities and explanations of experimental results.

In experiments previously mentioned lytic viruses had been obtained on a number of occasions by subculturing various strains with the indicator strain H10. When this strain was subcultured with the supernatant fluid from previous subcultures of the same strain virus preparations were obtained after a few passages which readily lysed the parent strain. It is evident, therefore, that when the indicator strain H10 was subcultured with another strain and a virus attacking H10 was obtained it could not be assumed that the virus originated from the second insusceptible strain, since the virus could have arisen by adaptation from a symbiotic virus originally present in H10 itself. There were differences in the properties of viruses obtained by subculturing H10 with different diphtheria strains. Some of these apparently had not originated from the indicator strain but from the strain incubated with it. As mentioned later, there is the further complication of the possibility of genetic recombination between the infecting and symbiotic viruses.

The adaptation of a symbiotic virus to attack its own bacterial host has been repeated in a number of cases, but it may be difficult to demonstrate directly in the case of a lysogenic strain which liberates only a few virus particles spontaneously, and there may be too few particles present to include any mutants capable of the required adaptation. In these cases it is necessary first to propagate the lysogenic virus on a susceptible strain and, when a potent virus preparation has been obtained, to adapt the filtrate to the original parent strain. The disadvantage of this indirect procedure is that the susceptible bacterial strain used for the propagation may itself be lysogenic and liberate virus particles which intervene and complicate the autoadaptation process, rendering the origin of the resultant adapted virus preparation obscure. When it is possible to adapt a virus directly to attack its own parent bacterial strain the origin is indisputable.

Spontaneous autoadaptation

There is abundant evidence that autoadaptation of symbiotic viruses to attack their own hosts occurs spontaneously. For example, when old cultures of the strain H10 are freshly subcultured after a long interval the cultural appearance is sometimes abnormal and there is little growth in the second subculture. Spontaneous lysis has occurred and the virus susceptibility of the surviving cells is markedly subnormal. The explanation of these phenomena lies in spontaneous autoadaptation during storage of the old culture. Stored cultures are not in a state of completely suspended animation, and from time to time some of the lysogenic bacterial cells burst liberating free virus particles. An occasional mutant virus, perhaps less than one in a million of the virus particles present, is capable of adaptation to attack the parent bacterial strain. Once liberated these mutant viruses will multiply by infecting the host cells, whilst the normal virus particles are unable to proliferate since there is not a susceptible host present. In old stock cultures therefore there will be a gradual increase in the proportion of mutant viruses. On first subculturing aged stock cultures of bacterial strains of this type the mutant viruses multiply at the expense of the host cells and filtrates from these frequently have pronounced lytic activity against normal subcultures of the same strain. These mutant viruses are often of relatively feeble activity, they are readily superseded and tend to constitute only an almost undetectable minority in normal cultures, but their presence can be detected by special experimental methods as described in the next section.

Inhomogeneity of cultures

In a bacterial strain symbiotically infected with a virus (as indeed most strains are in the case of many bacterial species) the population of a culture containing hundreds of millions of cells is not completely homogeneous. Occasional cells contain virus particles differing from the remainder. Under ordinary cultural conditions this lack of homogeneity is not apparent since virus particles liberated from bursting cells infect any susceptible bacteria present and this has a homogenizing effect. As will be shown in a subsequent communication some viruses have a dominant effect and tend to overcome the effects of other less active ones under normal cultural conditions. If, however, this spread of virus infection through a culture is inhibited and individual members of the bacterial population are examined, the inhomogeneity can be detected. For successful attack on susceptible bacteria many viruses require the presence of divalent cations such as calcium (Bordet & Renaux, 1928) and substances interfering with free calcium ions inhibit virus activity (Stassano & de Beaufort, 1925). For this reason a number of diphtheria strains were subcultured in broth containing sodium oxalate so that virus multiplication was minimized, and then the subcultures were plated out on a solid medium on which a variety of colonial types can be distinguished readily. Heated blood agar plates containing tellurite can be used for this purpose, but much better differentiation is observed with laked blood agar plates containing 0·01 % sodium selenite (Hewitt, 1948). On this medium a variety of colonies differing in size, shape and colour occur. Some organisms yield red colonies, others white and others with red and white bands. A typical experiment of this type was carried out with a gravis diphtheria strain H874. The strain was subcultured alternately in oxalate broth and on selenite blood agar plates, selecting colonies of different types. Most of the colonies were of medium size uniformly red in colour and when examined were found to be indistinguishable from the original parent cultures. Among these colonies were a few larger colonies, umbonate in shape, the central dome being bright red with a white peripheral annulus. These 'jam tart' colonies (H874 JT) after several subcultures bred true and yielded a uniform colonial appearance when subcultures were plated out. The cultural characteristics were different from the original strain, growth in broth was poorer and less granular, producing a uniform turbidity. The original strain was virulent and lethal to guinea-pigs and in broth cultures toxin was formed, but the JT strain was completely avirulent and non-toxigenic, although starch was still fermented. The most interesting feature of the strain from the point of view of the present communication was that the virus it was carrying was different from that of the original strain. The parent strain was lysogenic and carried virus particles which attacked certain other strains, but the JT strain was symbiotically infected with a virus of different host range specificity which was capable of lysing the parent strain. It thus appeared to be a mutant virus which had arisen spontaneously from the virus particles carried by the parent strain. The bacterial cell carrying this mutant virus, when it divided, yielded daughter cells carrying the same mutant, but in normal broth cultures of the parent strain these would have been infected with the vigorous virus particles carried by most of the bacterial cells in the culture and the relatively feeble mutants would disappear. In the artificial conditions where virus multiplication and infection were inhibited by oxalate followed by segregation of the separate bacterial cells in the solid culture medium, it was possible for bacteria carrying mutant viruses to survive. When isolated in pure culture these aberrant strains survive and their characteristics have been maintained after many subcultures in normal broth. Some of these have been subcultured for 6 months without any apparent change or reversion.

Genetic recombination

It has already been mentioned that the behaviour of a virus preparation may depend, not only on its origin, but also on the bacterial strains upon which it has been propagated. Some effects of this on virulence will be described in a subsequent communication but the host-range aspect should be considered here.

Some experiments with the three gravis diphtheria strains H 868, H 873 and H 874 are of interest in this connexion and are summarized in Table 1.

Table 1. *Effect of propagating strain on host-specificity of viruses*

Virus preparation		Lytic effect on susceptible strain		
From strain	Propagating strain	H868	H873	H874
H873	H868	+	0	+
H873	H874	+	0	+
H874	H868	+	+	0
H874	H873	+	+	+

Virus preparations obtained from strain H874 lyse the strains H868 and H873, whilst the virus from strain H873 lyses strains H868 and H874. When H874 viruses are propagated on strain H868 (giving virus 874/868) the filtrate lyses both H868 and H873 but not the original parent strain H874. However, when H874 viruses are propagated on strain H873 the resultant filtrate (874/873) lyses not only strain H868 and H873 but also H874, the parent strain from which the virus preparation was originally obtained. Moreover, when strain H868 is treated with the virus 874/873 the resistant culture which grows out is found to consist of organisms carrying a virus, which can be liberated by ultraviolet irradiation. The virus filtrate thus obtained is found still to be active against all three strains H868, H873 and H874. It would appear that genetic recombination had occurred between the infecting virus from strain H874 and the symbiotic virus carried by strain H873 and that this genetic recombination persisted when the virus was propagated on strain 868, and also when it entered into symbiotic relationship with H868. The possibility has not been absolutely excluded that the virus preparation is a mixture of viruses and not a genetic recombinant. It is interesting to note that when the virus from H873 is propagated on strain H874 the resulting virus preparation still has the properties of H873 virus and is not a genetic recombinant. Regardless, however, of the final theoretical explanation of the phenomenon the practical point remains that the properties of H874 viruses depend upon the strains upon which they are propagated.

DISCUSSION

Among many species of bacteria, lysogenicity is very common and there is a growing volume of evidence that this widespread symbiotic infection with viruses plays a decisive role in controlling bacterial behaviour and evolution.

In attempting to elucidate the detailed mechanism of the virus control of bacterial variation complications are encountered. Bacterial viruses can be propagated only in bacterial cells and it is becoming increasingly obvious that the host on which a virus is propagated can affect the properties of the propagated virus. The virus preparation obtained by propagation on a given strain may be profoundly different from the original virus used as the seed. It is well-known that passage of a virus through a new host may modify its host-specificity and this effect has been noted in the case of animal and plant viruses as well as bacterial viruses. The important point that it is wished to emphasize here, however, is that viruses can often be readily adapted to attack the parent bacterial hosts with which they have entered into a symbiotic relationship.

Normally a lysogenic strain is resistant to the attack of the virus with which it is symbiotically infected; otherwise virus particles liberated by an occasional bursting cell would immediately lyse the parent culture. It is now found, however, that symbiotic viruses can, in fact, be adapted to attack the parent strain. This autoadaptation is quite different from the lysis induction process studied by Lwoff (1953). It has been shown by Lwoff *et al.* (1950) that a lysogenic culture may be induced by ultraviolet irradiation or other treatment to lyse and liberate virus particles. The virus liberated, however, does not attack the parent strain and the treatment has merely induced the cells either to produce complete virus particles from previous intermediate forms (Lwoff, 1953) or, at any rate, so to modify the host-virus relationship that the host cell bursts and liberates free virus particles. Boyd (1951), in the cases he has studied, finds evidence of the existence of two kinds of virus mutants, one kind being lytic and the other, instead of causing susceptible cells to lyse, tends to form stable symbiotic infections of the bacteria attacked. In neither the induction phenomena studied by Lwoff nor the mutations of Boyd, however, is there change in host-range specificity and the liberated virus particles are not able to lyse bacterial cells carrying the same virus. For free virus particles to cause lysis of bacterial cells carrying the same virus their host specificity has first to be modified or adapted.

As already indicated, the term adaptation is used here, not in the narrow sense of a non-hereditary cytoplasmic alteration, but with the broad meaning indicated by Stanier (1953). Adaptation therefore is used to describe the totality of processes by which the specificity of a virus preparation is modified to enable it to multiply in a new host. This may well involve both genetic and environmental factors, including perhaps the selection of an occasional virus mutant capable of gaining entry into bacterial cells which resist the attack of most members of the virus population. Once the parent virus has gained entry the daughter virus particles produced by replication processes may be modified, both by the synthetic mechanisms of the host cell and by other virus particles or proviruses already present. The experiments described in this communication and other preliminary observations suggest that factors influencing virus specificity are very complex (Burnet & Lush, 1936). Propagation of virus particles on different hosts may yield virus preparations of totally different properties and specificities, the factors involved including mutation, selection, genetic recombination with other viruses and environmental influences.

There are several practical consequences of this autoadaptation phenomenon. When two bacterial strains, A and B, are cultured together and a virus preparation results which lyses one of them, say A, it is not safe to assume that it necessarily arises from the other strain, B, since it may arise by autoadaptation of the virus carried by A itself.

As envisaged previously (Hewitt, 1953) it is possible to have a completely closed evolutionary system consisting only of a lysogenic bacterial strain and the virus it carries. By autoadaptation of the virus to attack the host strain an evolutionary chain is initiated. The parent strain is attacked by the mutant virus and the resistant organisms only survive. In turn the virus may mutate to attack this resistant strain and so on, there being no limit to the evolutionary steps that can occur. This process has been simulated in the laboratory by adapting lysogenic viruses to attack the host strain, isolating the consequent resistant bacterial strain and re-adapting the virus to attack this new strain. Steps in the chain can, however, be recognized in cultures which have not been subjected to artificial interference. In ordinary bacterial cultures virus mutation occurs and bacterial variants resistant to these mutants arise but the bacterial population remains fairly homogeneous because the viruses present reinfect the bacterial cells and a given virus tends to become dominant and overwhelm other less vigorous rivals. It is possible, however, to inhibit this homogenization process by adjusting the culture medium to restrict virus multiplication and infection. One method which has been found successful is to rid the medium of calcium ions which are necessary for virus multiplication and then to plate out the culture on solid medium on which viruses cannot be transmitted from a bursting cell to other bacteria in the culture. By alternate subculture in calcium-deficient liquid media and on a differential solid medium it is a simple matter to select from ordinary bacterial cultures variant bacteria carrying viruses different from the main bulk of the bacterial population. These variant bacteria frequently differ in important biological properties and variations in virulence, metabolism, colonial form, morphology, etc., have been found. The viruses they carry vary and some may be found which attack the parent culture, but if these viruses are of a recessive character they will tend to disappear in ordinary cultures where dominant viruses overcome their effect. Changes in cultural conditions may, however, disturb the balance between dominant and recessive viruses and, for example, in old cultures a complete and spontaneous change in cultural characters is sometimes observed on subculture. The explanation of these apparently spontaneous variations remains obscure until the effect of bacterial viruses is taken into account.

The phenomenon of autoadaptation probably accounts for the results of Smith & Jordan (1931) and Stone & Hobby (1934) who observed lytic action on one diphtheria strain when it was subcultured with a high proportion of other strains. At times this strain showed a 'suicidal tendency' and died out. This strain showed marked variability, and it seems probable that it was lysogenic and the virus it carried was readily adapted to lyse the parent strain. In the case of staphylococci Fisk (1942) found a number of very variable strains which after storage liberated a virus which lysed cultures isolated when plating out the parent strains. Autoadaptation of the carrier virus would account for Fisk's conclusion that variation in stock cultures 'may be an indication of alteration in the normal relationship of phage to bacterium'. Oeding (1950) has described colonial and other variants in diphtheria cultures including 'an eroded bacteriophage-like appearance' in the colonies which may readily be explained on the basis of virus autoadaptation.

A further property of viruses may complicate the issue when more than one virus is present. Evidence has accumulated that sometimes when a lysogenic bacterial strain is attacked by a virus to which it is susceptible, the virus particles liberated when it bursts may differ from the infecting particles and may be hybrids produced by genetic recombination between the infecting virus and the symbiotic virus carried by the original strain. Virus preparations with the host specificity of the two strains are obtained consistently from some gravis diphtheria strains.

Hershey & Rotman (1949) report genetic recombination between mutants of *Escherichia coli* T_2 bacteriophage simultaneously infecting bacterial cells. This result was confirmed by Visconti & Delbruck (1953), and a similar result obtained with T_4 bacteriophage by Doermann & Hill (1953). It is assumed that during multiplication of the virus in the cell there is random mating of the particles giving rise to the hybrid viruses. As far as is known there has, however, been no report of hybridization between virus particles attacking a cell from outside and virus particles already present in lysogenic cells. In the present experiments, however, when the virus (a) from one strain (A) infected another diphtheria strain (B), which was already carrying a virus (b), the virus preparation from the lysed cells lysed both strains A and B and behaved as if it were the hybrid ab with the host specificities of both viruses. On the other hand, when virus b was propagated on strain A the resulting virus preparation did not behave as the hybrid ab but like the original virus b with the admixture of relatively few particles of a from the lysogenic cells. More work is required to establish definitely the existence of this recombination, since recent experiments indicate the presence in the two strains, not of two viruses a and b only but four (a_1, a_2, b_1 and b_2). These complications provide the explanation of both the difficulties and value of phage typing methods. The intervention of lysogenic virus in the preparation of typing phages makes reproducible results difficult to obtain (Smith, 1948), but increases the possibility of differentiating strains of diverse origin (Craigie & Yen, 1938; Williams & Rippon, 1952).

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Mechanism of Virulence Transfer by Bacterial Viruses

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SUMMARY: Further experimental evidence is described to support the concept that bacterial viruses exert a controlling effect on bacterial variation and evolution. A number of strains of diphtheria bacilli, both mitis and gravis, have been found to be carrying viruses capable of converting a susceptible avirulent diphtheria strain to full virulence and toxigenicity. The virus-resistant strains thus converted to virulence retain this property when subcultured and the change can be effected *in vivo* as well as *in vitro*. The mechanism of virulence transfer has been investigated, and it appears that this virus-controlled conversion to virulence cannot be explained on the basis of the selection of virulent mutants already present in the avirulent culture. Also lysogenicity in a diphtheria strain does not itself imply virulence, since some viruses which attack the avirulent strain give rise to resistant cultures which are lysogenic and carry the infecting virus but they remain avirulent and non-toxigenic. It remains possible that the infecting virus transfers genetic properties such as virulence from the original virulent host bacterium to the infected strain. Selection by the infecting virus is also involved, and when a bacterial strain becomes infected with a mixture of viruses one of these becomes dominant in any definite cultural condition, infects all the cells in the culture and thus produces a 'homogenizing' effect. When precautions are taken to limit this spread of virus throughout the bacterial population it is found that ordinary laboratory cultures of 'pure' bacterial strains contain variant bacteria with distinctive properties. These when isolated are found to be infected with mutant viruses. The origin of the virus affects its virulence-conveying property; for example, virulence has not yet been transferred by a virus originating from an avirulent strain. On the other hand, some viruses carried by virulent diphtheria strains have been found to be incapable of conveying virulence, although they are able to render the avirulent culture lysogenic. Not only the origin but also the subsequent history of a virus affects its virulence-conveying power. One virus preparation was found to retain its virulence-conveying ability indefinitely when propagated on the avirulent strain, whilst after one passage through a susceptible virulent strain the ability was lost.

Interpretations of the phenomena associated with lysogenicity are complicated by the frequent occurrence of bacteria simultaneously infected with several distinct viruses and by the possibility of hybridization effected by genetic recombination.

The intervention of bacterial viruses can have decisive effects on the behaviour of bacterial cultures, and it has been shown that these individual phenomena can be related to a general scheme for the control of bacterial evolution by viruses. Some of the methods by which viruses are adapted or evolved to attack bacterial strains are dealt with in the previous communication (Hewitt, 1954), and attention is now directed to the effects of viruses on the biological behaviour of diphtheria bacilli.

A number of workers describe the existence of viruses attacking diphtheria bacilli (d'Hérelle, 1918; Botez, 1921; Blair, 1924; Fejgin, 1925; Klosterman & Small, 1928; Smith & Jordan, 1931; Stone & Hobby, 1934; Keogh, Simmons & Anderson, 1938; Toshach, 1950; Fahey, 1952; Thibaut & Fredericq, 1952), and in a previous communication it was shown (Hewitt, 1952) that mitis type III strains of *Corynebacterium diphtheriae* (Hewitt, 1947, 1948) could be sharply differentiated into two subtypes according to their susceptibility to a virus lysing one of the strains of this serological type. Type IIIA strains were completely resistant to this virus whilst type IIIB were susceptible. Strains of type IIIA were fully virulent and produced diphtheria toxin in broth cultures, whilst type IIIB strains were completely avirulent. Addition of the virus to some type IIIB strains rendered them fully virulent. This virulence conversion is not an isolated phenomenon, and other workers (Freeman & Morse, 1952; Parsons & Frobisher, 1951; Groman, 1953; Barksdale & Pappenheimer, 1954), using a bacteriophage preparation obtained from Toshach (1950), have isolated virulent cultures from a strain originally found to be avirulent.

The mechanism by which this change from avirulence to virulence is effected is uncertain. Recently a number of gravis diphtheria strains have been encountered which were carrying viruses which converted completely avirulent gravis strains to full virulence, and the opportunity has been taken of studying the mechanism of the phenomenon in the light of recent observations of host-virus relationships.

MATERIALS AND METHODS

Both recently isolated diphtheria strains and those kept in laboratory subculture or in the dried state were used; the methods of characterization and typing were those previously described (Hewitt, 1947). Viruses were obtained from lysogenic diphtheria strains, and methods of propagation and testing were as previously described (Hewitt, 1952, 1954), except where otherwise stated.

For examining toxigenicity a qualitative indication may be obtained by observing zones of precipitate formation in agar plates containing diphtheria antitoxin which have been seeded with cultures of the strains concerned (Elek, 1948; Ouchterlony, 1949; Groman, 1953). However, zones of non-specific flocculation occur (Pope, Stevens, Caspary & Fenton, 1951) and the results are often difficult to interpret. On the other hand, a quantitative measure of toxigenicity is given by intracutaneous titration in albino guinea-pigs against standard antitoxin of culture filtrates, using standardized culture media. Virulence was tested by the lethal effect of intracutaneous injection into guinea-pigs of 0·2 ml. of culture washed off Loeffler slopes. Death usually occurred in 24–48 hr. and was prevented by intraperitoneal injection of diphtheria antitoxin at the same time as the culture in control animals. As found previously (Hewitt, 1948) there was no discrepancy between virulence and toxigenicity tests. Cultures found to kill guinea-pigs produced in broth cultures toxin giving a skin reaction in albino guinea-pigs and those producing skin-reacting toxin also killed guinea-pigs.

RESULTS

Virulence transfer

The virulent gravis diphtheria strain H870 was found to be lysogenic and symbiotically infected with a virus which was liberated when cultures were irradiated with ultraviolet light. The virus attacked the avirulent gravis strain H868 producing areas of lysis on serum agar plates. Broth cultures of strain H870 were irradiated with ultraviolet light and incubated for 6 hr. and then filtered through a collodion membrane of average pore diameter (A.P.D.) 0·7 μ. A sample of broth was inoculated with the avirulent strain H868, 0·5 ml. of the virus filtrate was added and the culture was incubated at 37° for 18 hr. A resistant culture had grown out and this was subcultured in broth for resistance and virulence tests. When plated out on serum agar the strain was now found to be resistant to the action of the H870 virus and no lysis was observed. Although the original culture H868 was avirulent and non-toxigenic the resistant strain 868 R 870 was virulent and produced 0·7 unit/ml. of diphtheria toxin. Four other gravis strains were also found to be carrying viruses capable of converting the avirulent strain to virulence and toxigenicity. The toxin produced by the strains made resistant to the four viruses ranged from 0·3 to 1·0 unit/ml.

Effect of ultraviolet irradiation

The virus preparation used to transfer virulence had been obtained after ultraviolet irradiation of strain H870. In order to eliminate the possible effects of ultraviolet light a virus preparation was made from cultures not irradiated. 18 hr. broth cultures of strain H870 were filtered and the filtrate was found to contain virus. This virus added to the avirulent strain H868 gave rise to a resistant strain which was virulent and toxigenic. The toxin produced in broth (0·6 unit/ml.) was approximately of the same potency as that produced by the strain made resistant to the ultraviolet-liberated virus (0·7 unit/ml.). The same result was observed with other viruses (see Table 1).

Table 1. *Toxigenicity of resistant cultures from avirulent strain* H868 *treated with viruses*

Strain*	Virus obtained from strain no.	Toxin (unit/ml.)
H868	0	0
868 R 156	H156	1·0
868 R 869	H869	0·3
868 R 870	H870	0·7
868 R 874	H874	0·7
868 R 875	H875	0·6

* The same convention is used for naming resistant strains in Tables 1, 4, 5 and 6. The first number refers to the original strain and the second number indicates the strain from which the virus preparation was obtained. Thus 868 R 156 signifies a culture obtained from the original strain 868 and made resistant to a virus preparation obtained from strain 156.

Nature of virulence-transferring agent

The cumulative evidence of qualitative and quantitative experiments to be described in later sections supported the view that the agent transferring virulence from the virulent to the avirulent strain was the specific virus or bacteriophage. Preparations with no lytic activity failed to induce virulence and strains not developing resistance to the virus remained avirulent. It remained a possibility, however, that the agent might not be a virus particle but a chemical compound similar to the pneumococcus transforming principle of Avery, Macleod & McCarty (1944). A purified virus preparation free from other bacterial products was therefore prepared. Virus obtained from strain H 874 was first filtered through collodion membranes of A.P.D. 0·7 μ. The cell-free filtrate thus obtained was then filtered through a collodion membrane of A.P.D. 0·07 μ. This membrane held back the virus particles but allowed products of bacterial metabolism, etc., to pass into the filtrate. The virus retained on the membrane was washed with broth and then the membrane was removed from the filter and the virus was resuspended in broth. This purified virus preparation retained both its original lytic activity and its virulence-transferring property. The avirulent strain treated with the ultrafiltrate remained avirulent whilst when treated with the purified resuspended virus it became virulent and produced in broth 0·6 unit/ml. of toxin. All attempts to separate the virulence-transferring principle from the virus particles have been unsuccessful.

Virulence transfer in vivo

If the transfer of virulence is possible only in laboratory cultures and does not take place *in vivo* the phenomenon might have only theoretical interest. Attempts were therefore made to demonstrate virulence transfer in the living animal. Injections were made intracutaneously. In the first case 0·2 ml. of the avirulent culture was injected, in the second 0·2 ml. of the purified virus preparation H 874, and in the third case 0·1 ml. of the avirulent culture followed by 0·1 ml. of the virus preparation. No reaction was produced by the avirulent culture or by the virus preparation alone, but when both were injected a typical diphtheritic skin reaction appeared and this proceeded a few days later to a necrotic area and scab. The avirulent culture had thus been transformed *in vivo* into the toxigenic state.

Permanency of virulence conversion

In order to test whether the conversion of avirulent strains was permanent or transient strains made virulent by various viruses (156, 870, 874 and 875) were subcultured in broth several times, then on Loeffler serum slopes, then in broth and were then plated out on blood agar plates. Single colonies were picked off, subcultured in broth and then tested for toxigenicity. In the case of each of the four virus-resistant strains toxigenicity was retained unimpaired. Since this experiment was completed some of the resistant strains have been subcultured many times over a period of months but no reversion to avirulence has occurred. An exception was found in the case of strains infected with virus 869 which reverted to avirulence after 6 subcultures.

Virulence transfer by selection

Various mechanisms can be postulated (Hewitt, 1953) to account for the effect of viruses on bacterial variation, and the first of these is by the selection of resistant mutants. The original avirulent culture might be considered as consisting of mainly avirulent cells and perhaps one in a million being a virulent mutant. The virus may then lyse all the normal avirulent cells leaving intact only the occasional virulent mutant cells which will then grow out and yield a fully virulent culture. The delicacy of the toxigenicity test is such that the presence of one virulent cell amongst a thousand avirulent cells could readily be detected. To test whether such a selection was taking place, a culture of the avirulent strain containing approximately 5×10^7 cells was treated with approximately 5×10^7 particles of virus H874. The mixture was incubated for 10 min. to ensure that the bacterial cells had been infected with virus. Serial tenfold dilutions in broth of the virus-infected culture were then made and the dilutions were incubated overnight. Next morning each tube was examined for bacterial growth and virus activity and the toxigenicity of the strains was determined after subculture; the results are shown in Table 2.

Table 2. *Toxigenicity transfer in diluted cultures (multiplicity of infection; virus particles/bacterial cells* = 1)

No. of infected cells	Bacterial growth	Virus multiplication	Toxin production (unit/ml.)
5×10^5	+	+	0·4
5×10^4	+	+	0·5
5×10^3	+	+	0·3
500	+	+	0·3
50	+	±	0·6
5	0	0	0
0	0	0	0

It will be seen that in cultures originally containing five infected bacterial cells or less there is no growth, presumably because any bacteria originally present will have been lysed. In cultures containing fifty cells or more, however, some bacteria survive attack by the virus and grow out. In each culture in which there was bacterial multiplication the bacteria had become fully toxigenic, although the most diluted culture in which growth occurred contained only fifty bacteria originally. This renders unlikely the hypothesis that the virus converts the culture to virulence by the selection of occasional virulent mutants since the presence of one virulent mutant cell in fifty of the original population would have been readily detected. A further experiment was now performed in which there were insufficient virus particles to infect more than one-tenth of the bacteria present. Dilutions of the avirulent culture were made as before and incubated. The results are summarized in Table 3.

To the avirulent culture was added the same virus preparation as above in the approximate ratio of virus particles to bacterial cells of 0·1. Tenfold dilutions were made as before and these were incubated for 24 hr. The toxigenicity of the virus-treated bacteria was examined after they had been subcultured and the results are summarized in Table 3.

Bacterial multiplication occurred in all the cultures originally containing any bacteria, and virus multiplication occurred in cultures containing fifty or more virus particles, but when five or less virus particles were present they failed to multiply. Toxigenicity was conferred in all cultures originally containing fifty or more virus particles. In cultures originally containing less than fifty virus particles full virulence was not developed but with this low multiplicity of infection (one virus particle to ten bacterial cells) it is uncertain whether there was insufficient virus multiplication to infect all the bacteria or whether some virus particles fail to confer virulence. At any rate the final culture was a mixture of virulent and avirulent cells and continued subculture of partially virulent cultures resulted in the conversion of the strain to full virulence or to complete avirulence by a virus-controlled homogenization process commented upon in a later section.

Table 3. *Toxigenicity transfer in diluted cultures (multiplicity of infection; virus particles/bacterial cells,* 0·1)

No. of bacterial cells	No. of virus particles	Bacterial growth	Virus multiplication	Toxigenicity (unit/ml.)
5×10^5	5×10^4	+	+	0·4
5×10^4	5×10^3	+	+	0·6
5×10^3	5×10^2	+	+	0·5
500	50	+	±	0·1
50	5	±	0	0
5	0	±	0	0
0	0	0	0	0

Viruses not transferring virulence

So far all the viruses described which attacked the avirulent strain were found to convey virulence. These viruses are, however, exceptional and most viruses are incapable of doing so. An example of this is the virus carried by the virulent gravis strain H873. Filtrates of this strain contain a virus which attacks the avirulent strain H868 and the amount of free virus can be increased by ultraviolet irradiation of the culture. When the virus is added to the avirulent strain some of the organisms are lysed, but some others survive and grow out. The resulting culture consists of organisms quite resistant to the action of the virus but they remain completely avirulent and produce no toxin in broth cultures. This experiment has been repeated a number of times with the same result and many other viruses have been found to be incapable of transferring virulence to susceptible avirulent strains.

Lysogenicity and virulence

Many resistant bacterial strains growing out after treatment of a susceptible culture with a virus and now found to be no longer sensitive to the lytic action of the virus are found to be lysogenic or symbiotically infected with the virus. It would thus seem possible that the virulence conveyed to the culture by the action of the virus may be the direct result of this lysogenicity. The question arises whether establishment of lysogenicity automatically confers virulence on an avirulent strain. When, however, the strains shown in Table 4 are examined it is found that not all virulent strains are lysogenic. When irradiated with ultraviolet light the supernatant fluids and filtrates of most of the virulent strains in fact contained viruses which lysed the original avirulent culture H868, but the strain which had been made resistant to the virus from strain H870, although still fully virulent, liberated no virus and showed no evidence at all of lysogenicity. This negative evidence is not, of course, conclusive, since a strain may be symbiotically infected with a virus without giving any evidence, and unless a virus is brought into contact with a susceptible strain its presence can go undetected. Much stronger evidence would be provided by showing that strains may be rendered lysogenic without developing resistance. This has now been done many times with a number of different viruses, and in fact relatively few viruses have been found which transfer virulence when they infect an avirulent bacterial strain. A typical example is the virus carried by the virulent gravis strain H873; this virus attacks the avirulent strain H868 and the resistant culture which grows out is found to be symbiotically infected with the virus, but the lysogenic culture remains completely avirulent and non-toxigenic (see Table 4).

Table 4. *Toxigenicity, lysogenicity and virus sensitivity of strains*

Strain no.	Toxigenicity (unit/ml.)	Sensitivity to virus			Lysogenicity
		H874	874/873	H873	
868 R 869 u.v.	0·7	0	+	+	+
868 R 870	0·6	0	+	+	0
868 R 870 u.v.	0·7	0	+	+	0
868 R 874 u.v.	0·3	0	+	+	+
868 R 875 u.v.	0·6	0	+	+	+
868 R 873 u.v.	0	+	+	0	+
868 R 869/873	0	0	0	0	+
868 R 869/873 u.v.	0	0	0	0	+
868 R 870/873	0	0	0	0	+
868 R 870/873 u.v.	0	0	0	0	+
868 R 874/873	0	0	0	0	+
868 R 874/873 u.v.	0	0	0	0	+
868 R 875/873	0	0	0	0	+
868 R 875/873 u.v.	0	0	0	0	+
868 R 874/873/868	0	0	0	0	+
868 R 875/873/868	0	0	0	0	+
874/868/873	0	0	0	0	+
H868	0	+	+	+	0
H873	1·5	+	+	0	+
H874	0·3	0	+	+	+
H875	1·3	0	+	+	+

Virulence transfer and genetic transduction

The conversion of avirulent strains to virulence might constitute a case of the transfer of genetic qualities, and evidence on this point was sought. The virus liberated by strain H874 attacks the avirulent strain H868 and the resistant lysogenic organisms which are not lysed are fully virulent, as mentioned above. This may be due to transfer of the hypothetical 'virulence gene' from the virulent organisms to the avirulent, together with the infecting virus. To test this view the virus was propagated on the avirulent strain, the cell-free virus filtrate being passaged in this way several times through the avirulent strain in order to rid it of any adhering genetic material.

In Table 5 are shown the results of serial passages of the virus from strain H874 through the avirulent strain H868. In each passage the virus was added to the avirulent strain H868 and after incubation for 18 hr. the resistant organisms which had grown out were subcultured and tested for toxigenicity. The remainder of the culture was filtered through a collodion membrane of A.P.D. 0·7 μ and the sterile filtrate was added to a fresh subculture of the avirulent strain. It will be seen that there is no diminution in the toxigenicity of the resistant strains obtained by treatment with successive serial transfers of the virus. Moreover, the resistant virulent strains produced were found to be carrying a virus which was liberated when the strains were irradiated with ultraviolet light, and this liberated virus was still capable of transferring virulence to the avirulent susceptible strain. The transfer of virulence would thus seem not to be a simple transfer of genetic material from a virulent to an avirulent strain, since the virus had been propagated on an avirulent host. It may be objected that since the avirulent cells were rendered virulent when treated with the virus the propagation was actually on a virulent strain, but this is not so since the propagation occurs in susceptible avirulent organisms and not on the resistant virulent mutants.

Table 5. *Effect of serial passage of virus through a virulent strain on virulence-transferring potency*

Strain	Virus	No. of passages through avirulent strain	Toxigenicity (unit/ml.)
H868	0	0	0
868 R 874	874 u.v.	0	0·5
868 R 874/868 (1)	874/868 (1)	1	1·1
868 R 874/868 (2)	874/868 (2)	2	0·6
868 R 874/868 (3)	874/868 (3)	3	1·1
868 R 874/868 (4)	874/868 (4)	4	1·5
868 R 874/868 (5)	874/868 (5)	5	1·0
868 R 874/868 (6)	874/868 (6)	6	1·1

At the same time the virus preparation obtained from H874 was propagated on another susceptible gravis strain H873 which is already virulent. The virus preparation thus obtained (874/873) was found to be still capable of attacking the avirulent strain H868 and the resistant mutants (868 R 874/873) were examined and found to be lysogenic and carrying the infecting virus (874/873), but they remained avirulent. Passage of the virus on the virulent strain H873 had thus deprived it of its virulence-transferring potency. The reason for this is at first sight obscure but it was found, as reported in the previous paper, that propagation of the virus on strain H873 had altered the virus in several respects, notably in its host range specificity. The virus from H874 propagated on strain H873 was capable of attacking not only strains H868 and H873 but also the original host H874. The virus appeared to be a genetic recombinant of the two viruses carried by strains H873 and H874 respectively. As mentioned above, the virus carried by H873 is incapable of transferring virulence to the avirulent strain and the virus preparation 874/873 perpetuates this impotence, although it renders the avirulent strain lysogenic. When this virus is propagated on the strain H868 it retains its extended host range specificity and still fails to convey virulence.

In Table 6 are summarized the results of an experiment in which cultures of the avirulent strain H868 were treated with viruses liberated from the virulent strains H873 and H874 and propagated on one or more of the strains H868, H873 and H874. The virus-resistant cultures were isolated and tested for the susceptibility to lysis by the various virus preparations. The lysis susceptibilities of the parent strains H868, H873 and H874 are included for comparison. Although the virus susceptibility of the original strain H868 had been modified by treatment with each of the viruses all the resistant strains remained avirulent except 868 R 874/868.

Table 6. *Lysis susceptibility of resistant strains*

	Strain tested						
Virus	868 R 874/868	868 R 874/873/ 868	868 R 873/868	868 R 873/874	H868	H873	H874
874/868	0	0	+	0	+	+	0
874/873/868	+	0	+	0	+	+	+
873/868	+	0	0	0	+	0	+
873/874	+	0	+	0	+	0	+

+, marked lysis on agar plate. 0, no appreciable lysis.

Some of the obscurities involved in these results may be resolved as a result of recent experiments, which it is intended to report in detail in a later communication. It appears that the various diphtheria strains involved are symbiotically infected with several distinct viruses so that complications are involved in the interaction of different viruses and bacterial strains.

Effect of mixed virus preparations

It is becoming clear that homogeneous virus preparations may be rare in nature, and that mixtures of different viruses are of common occurrence. The interrelations of the different viruses may be decisive in determining the effects of mixed virus preparations. The virulence transfer experiments described in this communication suggested that virulent diphtheria strains such as H874 might be infected with a mixture of viruses, and further evidence on this point is now being accumulated. At the moment it is sufficient to describe a typical experiment with strain H874. Preliminary experiments with one particular virus preparation from strain H874 propagated on strain H868 indicated that its virulence-transferring potency was much less than its lytic effect. Out of each 100 virus particles causing lysis of the susceptible strain only one appeared capable of transferring virulence. To cultures of avirulent strain H868 containing about 10^6 bacterial cells/ml. were added serial dilutions of the virus preparation 874/868, the ratio of virus particles to bacterial cells ranging from unity down to 10^{-6}. Virus multiplication occurred in all the cultures and the resistant cultures which grew out were examined with the results shown in Table 7. In the tubes containing 100 or more virus particles the resistant cultures were found, after subculture, to be fully virulent, although in tube no. 5 only about 100 virus particles had been added to 10^6 bacteria, and of the 100 only a few of the virus particles were probably capable of virulence transfer. In cultures to which ten virus particles or less had been added the resistant cultures, although lysogenic, remained avirulent. The sudden cut-off in toxigenicity was very striking and was shown to be due to the dominant role of the virulence-transferring virus. Subcultures from tube 5 (containing the smallest number of virus particles capable of virulence transfer) were irradiated with ultraviolet light, liberating the virus particles from the lysogenic bacteria, and the sterile virus preparation after filtration through collodion membranes was found to be capable of transferring virulence to avirulent cultures in a dilution of 10^{-4}. This virulence-transferring virus was found to possess lytic action against the avirulent strain cultured from tube 6. On the other hand, the virus preparation obtained by irradiation of the tube 6 cultures did not lyse the cultures from tube 5 and did not transfer virulence to the avirulent strain H868. It now becomes clear why the first five cultures in the experiment were fully virulent whilst the last two were completely avirulent. The last two cultures failed to develop virulence, of course, because the only virus particles present were of the kind not capable of transferring virulence. In the first five cultures, however, some of the bacterial cells became infected with 'avirulent' virus, whilst a few were infected with 'virulent' virus. The final culture is not, however, a mixture of the two resistant strains since, as has just been shown, 'virulent' virus liberated from the infected cells will lyse avirulent bacteria, whilst the 'avirulent' virus cannot attack the virulent cells. This illustrates an important principle of the control of bacterial evolution by viruses which will be discussed later, namely the homogenizing effect. Instead of a culture consisting of a mixture of bacterial cells infected with different viruses, there is generally one virus type which is dominant under any given cultural conditions, and this virus will attack different infected cells and 'homogenize' the culture.

Table 7. *Mixed virus effect on toxigenicity transfer*

Tube	Virus particles	Multiplicity of infection: Virus particles/bacterial cells	Virus multiplication	Toxigenicity (unit/ml.)
1	10^6	1/1	+	0·3
2	10^5	1/10	+	0·3
3	10^4	$1/10^2$	+	0·4
4	10^3	$1/10^3$	+	0·3
5	10^2	$1/10^4$	+	0·3
6	10	$1/10^5$	+	0
7	1	$1/10^6$	+	0

Effect of viruses on virulent strains

The following virus preparations which attacked the virulent strain H874 were added to it and the resistant cultures isolated: 869, 873, 873/868, 873/874, 874/873, 874/873/868, 874/868/873. In every case the resistant cultures remained virulent and no evidence of loss of virulence development was found, even when isolated colonies from solid media were selected.

Virus mutants in colonial variants

In ordinary cultures, as shown in a previous section dealing with mixed viruses, there is a tendency to a 'homogenizing' effect produced by the spread of a virus infection throughout the culture. This 'homogenizing' effect occurs, although mutant viruses probably arise and might be expected to produce a mixture of bacterial variants. One or other of the mutant viruses assumes a dominant position on account of its propagation characteristics, etc. This 'homogenization' can be prevented by inhibiting virus multiplication and adsorption, followed by selection of variant colonies on selective solid culture media.

As shown in a previous communication (Hewitt, 1954) mutants can be isolated from ordinary laboratory cultures by alternate subculture in oxalate broth and selenite blood agar. This technique was applied to the well-known Park-William 8 strain of diphtheria bacillus which is widely used for the production of highly potent toxin. The original strain yielded toxin filtrates varying in potency from 40 to 100 units/ml. but colonial variants were isolated which produced no more than 0·3 unit/ml. and subculture under varying conditions failed to enhance this figure. As described in the previous communication, colonial variants were also isolated from the virulent H874 strain which were quite avirulent, and these variants were found to be carrying a mutant virus capable of attacking the parent strain. The mutant virus no longer conveyed virulence to the avirulent strain H868 and the effect on the parent strain H874 was investigated. Three mutant virus preparations were prepared from irradiated avirulent variant strains and added to cultures of H874, the virus resistant cultures were isolated and examined for toxigenicity. These were still virulent and when cultured in broth produced toxins of potencies 0·3, 0·4 and 0·8 unit/ml. respectively. This result is not unexpected, since the virus carried by the parent strain appears to be 'dominant' and the 'recessive' avirulent virus is overshadowed by the virus carried by the main bulk of the bacterial population.

DISCUSSION

It is of some interest to confirm the possibility of converting avirulent diphtheria strains to virulent by means of bacterial viruses in the case of fresh series of diphtheria strains and viruses. In previous cases described only mitis strains have been rendered virulent, and it seemed possible that the phenomenon might not be of general significance. In the present series of experiments, however, gravis strains have been used exclusively and viruses carried by recently isolated strains as well as others isolated six years ago and kept in the laboratory since have proved to be equally capable of inducing virulence.

It is also significant that the virulence induction occurs not only *in vitro* but takes place with equal facility in the animal body, so that the phenomenon is likely to be of importance in natural infections.

Determination of the mechanism by which the virulence transfer takes place is of interest, apart from the virulence phenomenon itself, on account of the light it sheds on the general question of evolution control by bacterial viruses. It was first necessary to determine whether the virus particles themselves induced the virulence transfer, or whether some soluble transforming principle similar to those described by Avery *et al.* (1944), Boivin, Vendrely & Lehoult (1945), Austrian & Macleod (1949), Alexander & Leidy (1951) and Hotchkiss (1951). Since cell-free filtrates conveyed virulence it is evident that the contact of bacteria themselves is not essential as it was in the *Escherichia coli* genetic transfers of Tatum & Lederberg (1947). Virulence-transferring activity paralleled in each case the virus titre, and finally it was found that filtration through a membrane with an average pore diameter of 0·07 μ held back all the virulence-transferring potency with the virus particles, whilst the filtrate containing products of bacterial metabolism, etc. was quite inactive. All the potency remained with the virus particles when these were resuspended from the surface of the washed membrane. It was found that inactivation of the virus particles resulted in loss of the virulence-transferring potency. Transmission of the purified virus preparation by propagation on the avirulent strain resulted in multiplication of virulence-transferring powers, as well as virus particles. There is therefore no evidence that any chemical-transforming principle is involved in the virulence transfer.

Three mechanisms have been postulated by which viruses influence bacterial variation (Hewitt, 1953), and consideration of each of these reveals matters of interest.

(1) *Selection of resistant mutants*

When a virus is added to a specifically susceptible culture the normal organisms are attacked by the virus leaving intact only those mutant cells which differ from the others in resisting the lytic action of the virus added. The culture which grows out on incubation will therefore consist entirely of resistant mutants. It seemed possible, therefore, that the original avirulent culture consisted mainly of avirulent bacteria with an occasional virulent mutant. Addition of the specific virus would attack the normal avirulent cells, leaving intact only the resistant mutants, and if these were virulent the subsequent resistant culture would consist of virulent virus-resistant cells. That this simple explanation is improbable follows from experiments in which a limited number of avirulent bacteria were found capable of yielding virulent cultures. From populations as small as fifty bacteria, viruses produced virulent cultures. If the explanation were simple selection of resistant virulent mutants there must have been at least one virulent cell in fifty of the original bacterial population, whereas quantitative toxigenic measurements render impossible such a high proportion of mutants.

(2) *Development of lysogenesis*

When a virus is added to a specifically susceptible culture some of the bacterial cells, instead of being lysed, become symbiotically infected with the virus. The daughter cells continue to carry the virus and are resistant to the action of externally added virus of this type, whilst an occasional lysogenic cell will burst liberating a number of virus particles. It is possible that virulent diphtheria bacilli are all lysogenic and that lysogenicity *per se* confers virulence on symbiotically infected cells. This explanation again is contra-indicated by the experimental results. Some bacteria converted to virulence failed to show any evidence of symbiotic virus infection. This is essentially negative evidence, however, and is not as convincing as the demonstration that many strains symbiotically infected with some viruses and possessing all the characteristics of lysogenic strains remained completely avirulent and toxigenic. Virulence is conveyed only by certain viruses and many lysogenic strains are avirulent.

(3) *Genetic transfer*

Since the two simple mechanisms of virulence induction, namely, mutant selection and lysogenicity, fail to provide a complete explanation, the possibility remaining is that the virus infecting the cells transfers the 'virulence gene' from the original host of the virus to the new host. If this be the true explanation the mechanism is not a simple transfer, since a virus from a virulent lysogenic strain continued to carry the virulence-transferring principle after being propagated on an avirulent strain for many serial passages. On the other hand, when the same virus preparation was propagated on a virulent strain it lost its virulence-carrying potentiality in a single subculture. In this latter case the propagated virus differed in other respects from the original virus. It had an extended range of host specificity and was, probably, a hybrid virus formed by genetic recombination of the infecting virus and a lysogenic virus with which the propagating strain was symbiotically infected. The symbiotic virus carried by this second virulent strain was also incapable of transferring virulence. If, then, the virus inducing virulence in the avirulent strain does so by transferring genetic material from the original virulent host strain, this genetic material must form an integral part of the virus structure and be replicated exactly, even when propagated on the avirulent strain.

Zinder & Lederberg (1952) and Stocker, Zinder & Lederberg (1953) have described genetic transfer from one bacterial strain to another by means of a lysogenic virus but the same virus preparation could transfer any one of a large number of genetic properties. It appeared to be a matter of chance which genetic quality was transferred from one cell to another and the number of cells transformed with respect to any particular property was very low, perhaps only 1 in 10^5 or 10^6 cells. In the case of virulence transfer considered here the avirulent culture was found to be rendered fully virulent. This, of course, may be merely a difference in the efficiency of genetic transfer. Zinder & Lederberg (1952), however, visualize the carriage of genetic material as occurring by the following process. When the virus multiplies in a bacterial cell there is thought to be a fragmentation of nuclear material. This is in accordance with the morphological observations of Boyd (1949). During synthesis of the virus particles fragments of nuclear material are incorporated in the new virus particles. Hence different virus particles may carry different genes from the host cell. This interesting concept may provide an explanation of the adaptation of viruses to new host specificities. If the virus particles liberated from a single 'burst' of a bacterium are heterogeneous with respect to the genetic material they carry they may also differ slightly in their host specificity and some may be adaptable to attack fresh hosts, as has been found experimentally (Hewitt, 1952, 1954).

Experiments now in progress, which it is intended to report later, may lead to an explanation of some of the anomalies. It appears that the bacterial strains investigated are each carrying several distinct viruses and the relation of these to each other and to the bacterial hosts establishes a complex system which may account for the observed phenomena.

In addition to the theoretical implications with regard to bacterial genetics the virulence-transfer experiments shed some light on the manner by which viruses can be expected to influence bacterial evolution under natural conditions without laboratory control of the cultural conditions and purity of cultures. When a mixture of viruses, one capable of virulence-transfer and one not, was added to an avirulent strain the resistant culture, although at first a mixture, was found to yield a uniform fully virulent strain when it was subcultured. This proved to be due to the dominance of the virulence-carrying virus, and bacterial cells which had been converted to virulence were found to be carrying a virus capable of transforming to full virulence the cells infected with the avirulent virus. It was found that ordinary laboratory cultures which were normally homogeneous gave rise to variant cells carrying mutant viruses. Under ordinary culture conditions these variant cells are re-infected with the dominant viruses present in the culture and are either destroyed by lysis or made lysogenic with respect to the dominant virus. By special culture methods, however, in which virus propagation and infection were inhibited, it was found possible to isolate from normal laboratory strains variant cells with properties distinct from the main bulk of the bacterial populations. Variant cells, for example, were isolated from virulent diphtheria cultures which proved to be avirulent and with different colonial forms. These variants were found to be carrying mutant viruses capable of attacking the parent strain. The presence of these mutants may account for the possibility of adapting viruses to attack strains against which they were originally impotent, and in particular the parent strain normally resistant to the viruses it carries.

It is thus becoming possible to recognize experimentally the occurrence of various control processes exerted by viruses on bacterial evolution which have been predicted on theoretical grounds, and to explain some of the mutations in specificity governing the relations of viruses and bacteria.

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The Effect of certain Antibiotics and other Chemotherapeutic Agents on Lysogenicity and Virulence Transfer by Bacterial Viruses in *Corynebacterium diphtheriae*

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SUMMARY: The treatment of bacteria with antibiotics, chemotherapeutic agents or various chemicals influences the nature of the viruses with which the bacteria are infected. In the case of *Corynebacterium diphtheriae* many strains are infected with two or more distinct kinds of virus. Cultivation of these lysogenic strains in the presence of certain drugs may result in the removal of one of the viruses with a consequent alteration in the characters of the strain. Apart from changes in the host specificity of virus preparations yielded by the treated strains, these viruses may also differ in their capacity to transfer virulence to avirulent strains. For example, one diphtheria strain after treatment with certain antibiotics and chemicals developed the capacity to transfer virulence which it had not originally possessed. An avirulent strain after subcultivation in presence of certain drugs became susceptible to virulence-transfer from virus preparations not able to convey virulence to the parent strain. It appears that in lysogenic bacterial strains infected with two kinds of virus, one of the viruses may be more labile or easily removed than the other. Possibly the easily removable virus may be carried in the cytoplasm of the cell whilst the more stable virus may have some attachment to the nuclear apparatus of the bacterial cell. In one case investigated the stable virus was able to transfer virulence to an avirulent strain, whilst the cytoplasmic virus was not. It appears that there are two distinct mechanisms by which drugs may affect the viruses carried by bacterial strains: (i) by the selection of bacterial variants carrying a certain virus; (ii) by the preferential removal of the more labile viruses and inhibition of their multiplication. The results suggest criteria to be applied to the selection of potential chemotherapeutic agents for treating animal virus infections.

Complications in tracing the effects of bacterial viruses on bacterial variation and evolution were attributed in former communications to the presence of mixtures of viruses in lysogenic bacterial strains (Hewitt, 1954*a*, *b*). Further information on this point has now been sought by investigating the effects of various drugs on the nature of the virus preparations yielded by lysogenic strains. In these preliminary studies the substances chosen for study included antibiotics and other chemotherapeutic substances known to have bactericidal or bacteriostatic effects, various metabolic and enzyme poisons, competitive metabolite analogues and other compounds which may inhibit virus multiplication.

METHODS

The strains of *Corynebacterium diphtheriae* used, particularly a group of gravis strains, have been described previously (Hewitt, 1954*b*); the general experimental methods used were as before. Methods of obtaining drug-resistant strains have also been described (Hewitt, 1948*a*, *b*; 1950*a*, *b*; 1952).

Streptomycin was used in the form of the sulphate, crystalline erythromycin base was kindly supplied by Abbott Laboratories and propamidine di-*is*-ethionate by Messrs May and Baker. Other drugs were of the purest form obtainable and were sterilized by filtration of the solutions or by heating.

RESULTS

Effects of treatment with streptomycin

Growth of the diphtheria strains was inhibited by streptomycin sulphate in a dose of about 1 μg./ml., but after several subcultures in media containing graded dilutions of the antibiotic, resistant cultures were obtained which grew in broth containing 1000 μg. streptomycin sulphate/ml. (Hewitt, 1948 *a*). After several further subcultures in streptomycin broth followed by cultivation in plain broth the resistant strains were tested for toxigenicity, virus sensitivity and lysogenicity. The virulence of the resistant strains remained unaltered. Thus the streptomycin-resistant strain 868S remained avirulent, like the parent strain H868, and strains 873S and 874S remained virulent. The strains 873S and 874S remained lysogenic but there was a change in virus sensitivity from the parent strains H873 and H874, and a change in the characters of the viruses they were carrying. Virus preparations from the resistant and parent strains were obtained by ultraviolet irradiation of broth cultures followed by incubation and filtration through sterile collodion membranes. The behaviour of the strains and viruses is given in Table 1. It will be seen that 873S and 874S still carried viruses capable of lysing H868 but, whereas the virus preparation from the parent strain H874 lysed H873, the viruses from the streptomycin-resistant strain 874S did not. This suggests that the parent strain H874 was carrying two viruses, only one of which was capable of lysing H873, and that this virus disappeared during the development of streptomycin resistance. Confirmation of this follows from the fact that the virus preparation obtained from the parent strain H874 lysed the corresponding streptomycin-resistant strain 874S. The streptomycin-resistant strain had lost one of the viruses (the one attacking H873), and being no longer symbiotically infected with this virus was now susceptible to its action. Inspection of Table 1 fails to reveal any differences in the virus susceptibility of strain H868 when it is

Table 1. *Effect of streptomycin resistance of strains of* Corynebacterium diphtheriae *on lysogenicity*

Source of virus preparation (strain of *C. diphtheriae*)	Susceptible strains of *C. diphtheriae*					
	H868	868S	H873	873S	H874	874S
	Lysis produced					
H873	+	+	0	0	+	+
873S	+	+	0	0	+	+
H874	+	+	+	0	0	+
874S	+	+	0	0	0	0

+ = marked lysis on serum agar plate; 0 = no visible lysis.

made streptomycin-resistant and there are no apparent differences in the host specificity of the viruses carried by H873 and the streptomycin-resistant variant 873S; but alterations have, however, been produced, as shown in the next section.

Effect of streptomycin resistance on virulence transfer

Viruses from strain H874 convert the avirulent strain H868 to virulence, whilst H873 viruses do not (Hewitt, 1954*a*, *b*). In the next series of experiments virus preparations from both the parent strains and the streptomycin-resistant variants were added to the avirulent strain H868 and the streptomycin-resistant 868S. The virus-resistant strains which grew out were subcultured in broth to rid them of free virus particles and tested for toxigenicity. From the results given in Table 2 it will be seen that viruses from the strains H874 and 874S rendered both H868 and 868S fully toxigenic. On the other hand, there was a change in the behaviour of H873 viruses when the bacterial strain had been made streptomycin-resistant. Viruses from the parent strain H873 failed to convey toxigenicity to H868 or 868S, but viruses from the streptomycin-resistant strain 873S converted strain 868S to full toxigenicity. Only a trace of toxigenicity was conveyed to H868 by 873S viruses.

Table 2. *Effect of streptomycin resistance of strains of* Corynebacterium diphtheriae *on toxigenicity transfer by virus preparations*

Virus from (strain of *C. diphtheriae*)	Virus-resistant strain from	
	H868	868S
	Toxigenicity (units toxin/ml.)	
H873	0	0
873S	0·008	1·5
H874	0·6	0·6
874S	1·3	1·4

The explanation of the results from experiments in this and preceding communications is that the parent strains H873 and H874 each carry two distinct viruses and H868 one virus. During the development of streptomycin resistance, bacterial cells are selected which have each lost one type of virus, so that 873S and 874S are now each carrying only one type of virus and 868S is no longer lysogenic. The viruses carried by the parent strain H874 may be designated 874, 1(t^+) and 874, 2. Virus 874, 1(t^+) is dominant, as shown in the previous communication, and capable of transferring toxigenicity (t^+) to the avirulent strain H868. Virus 874, 2, is incapable of transferring toxigenicity to the avirulent strain but it is capable of lysing strain H873, unlike virus 874, 1(t^+). Since both the parent strain H874 and 874S carry virus 874, 1(t^+) both strains yield virus preparations which transfer virulence to H868, but only H874 carries virus 874, 2, hence the resistant strain 874S does not yield virus which lyses H873 and is indeed itself susceptible to lysis by 874, 2. In the case of strain H873, the dominant virus

873, 1, is incapable of transferring toxigenicity to the avirulent strain, but removal of this virus allows the weaker virus 873, $2(t^+)$ to infect other strains, and this virus is capable of conveying toxigenicity to the susceptible avirulent strain 868S. The strain H868 appears to be carrying a virus which hinders the conveyance of toxigenicity to the strain by the weak virus 873, $2(t^+)$ but not by the dominant virus 874, $1(t^+)$. Removal of the virus yields strain 868S which is no longer lysogenic, and there is therefore no hindrance to toxigenicity transfer even by the weak virus 873, $2(t^+)$. These conclusions are summarized in Table 3.

Table 3. *Viruses carried by lysogenic strains of* Corynebacterium diphtheriae

Strain of *C. diphtheriae*	Viruses carried	Dominant virus
H868	868, 1	868, 1
868S	0	0
H873	873, 1; 873, $2(t^+)$	873, 1
873S	873, $2(t^+)$	873, $2(t^+)$
H874	874, $1(t^+)$; 874, 2	874, $1(t^+)$
874S	874, $1(t^+)$	874, $1(t^+)$

Effect of various chemotherapeutic agents and other compounds

Some insight into the mechanisms by which streptomycin affected the viruses carried by lysogenic strains may be gained by studying the effects of various other substances. In each case strains were subcultured in broth containing serial dilutions of the given compound and subcultures were made from the culture which contained the highest concentration of the compound in which bacterial growth occurred. These serial subcultures were continued from 6 to 12 times and some increases in tolerance to the compounds were observed; in no case was the degree of resistance developed comparable to that obtained with streptomycin. Approximate inhibitory concentrations of the drugs in the case of various diphtheria strains are summarized in Table 4.

Erythromycin was found to be extremely inhibitory to diphtheria cultures, and the increased resistance developed when the organisms were subcultured in presence of this compound was comparatively slight, as observed with penicillin, and not marked as with streptomycin (Hewitt, 1948*a*). The increased resistance to propamidine was also of the same order as that observed previously (Hewitt, 1948*b*). It is perhaps of interest that sodium arsenite was much more inhibitory to diphtheria bacilli than was sodium azide, and that potassium cyanide and 2:4-dinitrophenol, although metabolic poisons, were relatively non-toxic.

The toxigenicities of the strains, derived from strains H868, H873 and H874, and by subcultivation in the presence of the various compounds listed in Table 4 were tested by growing cultures in standardized broth and titrating the culture filtrates against standard diphtheria antitoxin by intracutaneous injection in albino guinea-pigs. In no case was there any appreciable change

Table 4. *Development of resistance to certain compounds in strains of* Corynebacterium diphtheriae

Compound used	Initial inhibitory dose (μg./ml.)	Increased tolerance factor
Streptomycin	1	1000
Erythromycin	0·1	4
Propamidine di-*is*ethionate	5	4
Euflavine	10	4
Thiouracil	>1000	0
Barbituric acid	>1000	0
Arsenite	50	0
Azide	500	0
Cobaltous chloride	200	0
Cupric sulphate	200	0
Cyanide	200	2
2:4-Dinitrophenol	500	0

in toxigenicity except for a decrease in the case of the streptomycin-resistant strains and in the strain from H874 treated with cyanide.

Each drug-resistant strain was tested for its sensitivity to various virus preparations. The lysogenic viruses carried by the strains were investigated by ultraviolet irradiation of the cultures and testing the lytic activities of the sterile virus filtrates. There were no marked alterations of virus sensitivity of the strains H868 and H873 and the lysogenic viruses carried by these strains showed no change in host specificity after being treated with the various compounds listed in Table 4. The only alterations in virus sensitivity and in the nature of the viruses carried by strain H874 were those produced by streptomycin and already mentioned (Table 5). There were, nevertheless, changes in the characters of some of the lysogenic viruses as shown in the next section.

Table 5. *Lysogenic viruses carried by drug-resistant strains of* Corynebacterium diphtheriae *derived from strain* H 874

Virus derived from strain resistant to drug	Strain of *C. diphtheriae* H868	H873	H874	874S
	Lysis produced			
None	+	+	0	+
Streptomycin	+	0	0	0
Erythromycin	+	+	0	+
Propamidine	+	+	0	+
Euflavine	+	+	0	+
Thiouracil	+	+	0	+
Barbituric acid	+	+	0	+
Arsenite	+	+	0	+
Azide	+	+	0	+
Cobalt	+	+	0	+
Copper	+	+	0	+
Cyanide	+	+	0	+
2:4-Dinitrophenol	+	+	0	+

Virulence transfer from drug-resistant strains

The sterile virus filtrates obtained after ultraviolet irradiation of the drug-resistant strains were tested for their virulence-transferring activity by adding them to the susceptible avirulent strain H868. The virus-resistant strains which grew out after incubation overnight were subcultured twice in broth to ensure virus 'homogenization' (Hewitt, 1954*b*) before their toxigenicity was tested. The results are summarized in Table 6. It will be seen that viruses from the drug-resistant strains derived from H874 still conveyed toxigenicity whilst those from H873 still failed to convey toxigenicity except for the slight toxigenicity transferred by viruses from the streptomycin-resistant strain.

Table 6. *Toxigenicity transfer by viruses from drug-resistant strains of* Corynebacterium diphtheriae *to avirulent strain* H 868

	Toxigenicity conveyed by viruses from resistant strains derived from	
	H873	H874
Strain resistance	Units toxin/ml.	
None	0	0·6
Streptomycin	0·008	1·3
Propamidine di-*i*sethionate	0	0·5
Euflavine	0	0·9
Thiouracil	0	0·4

Table 7. *Toxigenicity transfer to avirulent strain* 868 S *of* Corynebacterium diphtheriae

	Toxigenicity conveyed by viruses from drug-resistant strains from	
	H873	H874
Strain resistance	Units toxin/ml.	
None	0	0·6
Streptomycin	0·3	1·4
Arsenite	0·6	0·9
Cobalt	1·4	2·4
Copper	0·8	1·1
Barbituric acid	0·01	1·5
Azide	0·003	1·4
2:4-Dinitrophenol	0·002	1·3
Erythromycin	0·0002	0·6
Propamidine di-*i*sethionate	0·0002	1·5
Euflavine	0·0002	1·4
Thiouracil	0	1·0

Different results, however, were obtained when the viruses were added to the streptomycin-resistant avirulent strain 868S; the results are summarized in Table 7. It will be seen that the H874 drug-resistant strains yielded viruses which conveyed toxigenicity as before, but the viruses from some of the H873 drug-resistant strains were also capable of transferring toxigenicity. H873 strains made resistant to arsenite, copper or cobalt as well as streptomycin converted the avirulent strain to full toxigenicity. Slight toxigenicity, in decreasing degree, was also conveyed by the viruses from H873 strains treated with barbituric acid, sodium azide, 2:4-dinitrophenol, erythromycin, propamidine di-*is*ethionate or euflavine. In each of these cases the compound had exerted an effect, in varying degree, of suppressing the non-virulence-conveying virus carried by H873 and thus enabled the virulence-conveying virus 2(t^+) to become dominant and effective.

Converted virulent strain

It was thought possible that an avirulent strain recently converted to virulence by treatment with a virus might be more susceptible to alteration or removal of its lysogenic virus. Strain H868 after treatment with viruses from H874 yielded a fully virulent and toxigenic strain 868R874. After frequent subculture in broth and on Loeffler slopes it was grown in streptomycin broth. After nine subcultures in this streptomycin broth its resistance to streptomycin had increased 1000 times. The streptomycin-resistant strain remained virulent and toxigenic (0·3 unit toxin/ml.) and when irradiated with ultraviolet light it liberated virus which was filtered through a collodion membrane and added to the avirulent strain H868. The virus-resistant strain which grew out was virulent and toxigenic (1·4 units toxin/ml.) The virus also conveyed virulence and toxigenicity (0·6 unit toxin/ml.) to the streptomycin-resistant avirulent strain 868S.

The converted avirulent strain (868R874) was also subcultured in increasing concentrations of propamidine di-*is*ethionate or thiouracil. In each case the toxigenicity was maintained (1·3 and 2·0 units toxin/ml. respectively) and the strains remained lysogenic. Thus in converting it to virulence the virus had formed a stable symbiotic infection of the avirulent strain and neither the lysogenicity nor the toxigenicity could be removed readily.

DISCUSSION

Streptomycin-resistant strains of *Corynebacterium diphtheriae* derived from lysogenic bacteria differ from the parent strains not only in antibiotic resistance but also in the viruses they are carrying. As a result of these experiments and others previously reported (Hewitt, 1954*a*, *b*) it is concluded that two virulent gravis strains of *C. diphtheriae* were each carrying two distinct viruses. In each case one of the viruses (t^+) was capable of transferring virulence and toxigenicity to a susceptible avirulent strain, whilst the other virus (n) was not. In one strain, H874, the toxigenicity-transferring virus t^+ was dominant whilst in the other strain H873 the virus incapable of transferring virulence, virus n, predominated. When the virus preparation from H874 was added to the avirulent strain the virus-resistant culture which grew out was fully virulent and toxigenic, whilst the resistant culture obtained by the action of the viruses from H873 on the avirulent strain remained completely avirulent and non-toxigenic, although it had been rendered lysogenic with respect to the infecting virus. On the other hand, when the streptomycin-resistant cultures 873S and 874S derived from the parent strains were examined they were both found to have lost the *n* virus but to have retained the virulence-transferring t^+ virus. This had little apparent effect on the H874 viruses since the t^+ virus had been dominant originally but, in the case of strain H873 the removal of the *n* virus allowed the t^+ virus to exert its effect and 873S viruses were capable of transferring virulence and toxigenicity to the susceptible avirulent strain, unlike the parent H873 viruses. The t^+ and *n* viruses from H874 differ in host specificity since only the *n* virus is capable of lysing strain H873. The streptomycin-resistant 874S strain, having lost its *n* virus, is now susceptible to lysis by viruses from the parent strain, which is infected with both t^+ and *n* viruses. From their behaviour it appears that the t^+ and *n* viruses of strain H873 differ from those of strain H874. In the case of the avirulent strain H868 the development of streptomycin resistance is accompanied by increased susceptibility to conversion to virulence. The parent strain H868 is rendered fully virulent and toxigenic by virus preparations which contain the dominant t^+ virus from H874, but not by the 'recessive' t^+ virus from H873 strains. The streptomycin-resistant avirulent strain 868S is, however, rendered fully virulent and toxigenic by the t^+ virus from H873 strains made resistant to streptomycin and various other compounds. Presumably H868 itself carries a virus which prevents transfer of virulence from 873 strains by the relatively ineffective 873 t^+ virus.

Other compounds besides streptomycin affect the viruses carried by lysogenic strains. In particular, arsenite, cobalt and copper salts are particularly effective in removing the dominant *n* virus from H873 so that the remaining t^+ virus is able to transfer virulence to the susceptible avirulent strain.

In seeking an explanation of the fact that it is always the *n* virus which is removed from the lysogenic strains by drug treatment it is perhaps significant to note that the more firmly established t^+ virus is the one capable of transferring toxigenicity to susceptible strains. The virulence or toxigenicity appears to be a genetic property permanently transferred to the strain and inherited by the daughter cells. It is possible, therefore, that the firmly established t^+ viruses are attached to the chromosomal or nuclear structures of the bacterial cell, whilst the relatively labile *n* viruses may be free in the cell cytoplasm. It may be that cases in the literature of loss of viruses from lysogenic strains (den Dooren de Jong, 1931; Clarke, 1952) may have occurred in the case of purely cytoplasmic viruses whilst the more firmly attached viruses with which attempts at removal have failed may be attached to the cellular genetic structure.

Although lysogenic bacteria generally transmit symbiotic viruses to all their progeny it is by no means certain that each of the infecting viruses forms part of the nuclear structure of the host bacterium. When the bacterial cell divides the viruses firmly attached to the nuclear structure would be transmitted to the progeny in indefinite series unless there was a change in the genetic structure of the cell corresponding to a mutation. In the case of viruses purely cytoplasmic in location transmission to the daughter cells would be much more haphazard and an occasional daughter cell would occur devoid of this particular type of virus.

Further evidence has accumulated, from the experiments reported in the present communication, on the mechanisms by which bacterial viruses may control bacterial variation and evolution. In particular it would seem that symbiotic viruses differ in their firmness of union with the bacterial cell; in the cases now studied the firmly attached viruses alone seem capable of transferring genetic properties to another bacterium. A further point concerns the dominance of certain viruses over others, but the dominant virus in a lysogenic bacterium is not necessarily the firmly attached virus; in one strain the more 'labile' virus predominated in its influence over the firmly attached 'nuclear' virus, whilst in another the 'nuclear' virus was dominant.

Bacterial viruses may be used for screening and selecting compounds for their potential value in the treatment of animal virus infections. It seems possible that effects in inhibiting bacterial lysis may be comparable to the prophylactic use of drugs for protection against virus infections. For the treatment of established animal virus infections the comparable phenomenon in the case of bacterial viruses is the 'cure' of lysogeny and the removal of lysogenic viruses. It may well be that, just as with the bacterial viruses, certain animal viruses may form more stable infections of their host cell than do other viruses. Eventually there may prove to be considerable differences in the efficacy of chemotherapy in different virus diseases for this reason.

Although streptomycin proved most successful in affecting the viruses carried by the lysogenic strains it is by no means certain that this reflects any direct activity against the viruses themselves. Streptomycin had no inhibitory effect on bacterial lysis by viruses. In the development of streptomycin-resistant strains a selection of bacterial variants had occurred and it may well be that the streptomycin-resistant strains differed from the parent strains mainly because the variants selected carried different virus combinations from the parent and not because the streptomycin had any marked direct effect on the viruses.

The mechanism by which streptomycin might be expected to select bacterial variants symbiotically infected with viruses attached to the nucleus but devoid of 'cytoplasmic' viruses may be visualized as follows. As already mentioned, in the case of the progeny of a bacterial cell infected with both 'nuclear' and 'cytoplasmic' viruses, all the daughter cells continue to carry the viruses firmly attached to the nucleus but an occasional daughter cell may be devoid of viruses present in the cytoplasm of the parent cell. A selective agent such as streptomycin which selects only one bacterial cell out of many million could not be expected to affect a virus attached to the bacterial nucleus but might well select for survival a cell devoid of the cytoplasmic virus infection. The streptomycin-resistant culture derived from this variant cell would then be devoid of the cytoplasmic virus. In the case of the other compounds tested there had not been the thousandfold increase in resistance, as with streptomycin, and hence not so narrow a selection of bacterial variants. In these other cases, therefore, the results may have been due to a more direct effect of the given compound on the virus infection.

It is interesting in this connexion to note that although Smadel, Jackson & Gauld (1947) found streptomycin of value against some rickettsial infections it has no effect on certain other virus diseases (Hamre & Rake, 1947). Although erythromycin has high bactericidal activity against diphtheria bacilli, it has little action on the symbiotic viruses; it did not give rise to highly resistant cultures, as did streptomycin, and hence did not select bacterial variants which carried different viruses. Hobson (1954) found that erythromycin did not affect the lysogenic pattern of staphylococci. Acridines are of limited value in some virus infections (Fitzgerald & Lee, 1946; Green, Rasmussen & Smadel, 1946; Hurst, 1948; Foster, 1948; Bourke, Robbins & Smith, 1952; Takemoto, Robbins & Smith, 1954). DeMars (1953) found that proflavine effected mutagenesis of viruses active against *Escherichia coli*; some compounds which induce the liberation of viruses from bacterial cells are also carcinogenic (Boyland, 1952).

Irwin & Irwin (1954) reported that an acridine compound reacted with nucleic acids; in the present experiments euflavine had only a very slight effect in suppressing one of the bacterial viruses. Propamidine di-*is*ethionate had only a slight effect on the lysogenic viruses of diphtheria, in accord with the results of Bourke *et al.* (1952) who found only a slight effect on lysis of *Escherichia coli.* In the case of purine and pyrimidine analogues Thompson, Price, Minton, Falco & Hitchings (1951) found some protection against vaccinia virus by phenoxythiouracil; Commoner & Mercer (1952), Jeener (1954), and Bawden & Kassanis (1954) found that thiouracil influenced some plant virus infections, but Matthews (1953) reported no effect. In the present work thiouracil had no effect on lysogenic diphtheria viruses; barbituric acid had a slight effect.

The other compounds investigated were chosen on account of their known effects on various bacterial enzymes and metabolic systems. Arsenite inhibits oxidative decarboxylation in the citric acid cycle and it was effective in ridding lysogenic diphtheria strains of one of the viruses carried. Azide and cyanide interfere with catalase activity and hence might enable peroxide produced in cellular metabolism to accumulate and to liberate lysogenic viruses, but the effect on lysogenic viruses was slight. Cobalt and copper salts may also lead to peroxide accumulation since thiol (sulphydryl) compounds yield peroxide by auto-oxidation in the presence of traces of these metallic ions. It is perhaps significant, therefore, that both cobalt and copper salts had a marked effect in removing a lysogenic virus from the diphtheria strains used here. 2:4-Dinitrophenol (DNP) causes an uncoupling of oxidative phosphorylation and might be expected to affect the synthesis of the nucleic acids of viruses. Both Heagy (1950) and Czekalowski (1952) found that DNP did inhibit certain bacterial viruses; in the present experiments it had only a very slight effect on the lysogenic diphtheria viruses.

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Pyruvate Oxidation by Extracts of *Tetrahymena pyriformis*

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SUMMARY: *Tetrahymena pyriformis* S has an enzyme system which splits pyruvic oxime. This permitted the use of hydroxylamine as non-enzymic acyl acceptor in assaying pyruvate oxidation enzymes in crude extracts. Removal of thioctic acid from crude preparations of tetrahymena pyruvic oxidase by alumina treatment resulted in decreased acyl group formation, but did not affect the rate of oxidation in the presence of ferricyanide. Purification of the oxidase removed both the oxime-splitting system and the enzyme which is necessary for removal of thioctic acid from enzymes by the alumina procedure.

Removal of thioctic acid from crude preparations of α-keto acid oxidases of tetrahymena decreases acylation activity as measured by following the formation of acyl-Coenzyme A by the hydroxamic method (Seaman, 1952, 1953). It was also observed that the addition of thioctic acid restored activity. Since under the conditions employed, hydroxylamine may perhaps be a rather non-specific indicator of acyl-Coenzyme A formation, it was desirable to extend the observations to assure that the observed effects are referable to the pyruvate system.

METHODS

Enzyme preparation. Tetrahymena pyriformis S was grown as previously described (Seaman, 1953). The carboys in which the organisms had grown for 72 hr. were placed in the cold room (4°) and the organisms allowed to settle overnight. The clear supernatant was siphoned off and the cells were concentrated by centrifugation at 850 ***g*** for 15 min. at 4°. After three washes in 0·01 M-phosphate buffer (pH 7·4) the concentrate was ground with twice its wet weight of quartz on an automatic grinder for 30 min. and extracted with 5 vol. of 0·01 M-phosphate buffer (pH 7·4) for an additional 30 min. The suspension was clarified by centrifugation at 5000 ***g*** for 15 min.

Preparations of pyruvic oxidase at three stages of purity were obtained by treating the turbid suspension according to the procedure of Jagannathan & Schweet (1952) for purification of the enzyme from pigeon breast muscle. Precipitation of the extract at pH 5·4 provided the crudest preparation examined (fraction 1). Later, freezing and thawing (fraction 2) and fractionation at pH 6·2 (fraction 3) provided purer preparations.

Assay procedures. The *ferricyanide manometric assay* (FCNM assay) was essentially as described by Jagannathan & Schweet (1952). The incubation mixture contained, in a volume of 3·0 ml., 100 μmole sodium pyruvate, 30 μmole $NaHCO_3$, 20 μmole $MgCl_2$, 200 μg. neutralized (pH 6·0) cocarboxylase, 100 μmole $K_3Fe(CN)_6$, and enzyme. Activity is expressed as μmole of CO_2

evolved/hr./10 mg. protein. Manometric runs were at 32° under CO_2. In this assay, analyses show a constant CO_2/pyruvate ratio of 2·06–2·23. The consistent difference between these values and the theoretical value of 3, indicates a non-oxidative side reaction of pyruvate, whose nature is yet uncertain.

The *hydroxamic acid assay* (HXMA assay) was carried out in mixtures containing, in 1·4 ml., 100 μmole pyruvate, 10 μmole $MgCl_2$, 100 μg. neutralized cocarboxylase, 20 units of Coenzyme A (Co A), 200 μmole tris(hydroxymethyl)-aminomethane (Tris) (pH 7·4), 10 μmole cysteine, 0·10 μmole diphosphopyridine nucleotide (DPN), 800 units of lactic dehydrogenase, 200 μmole hydroxylamine, and tetrahymena enzymes. Use of high hydroxylamine concentrations assured measurement of the *non-enzymic* acyl transfer from Co A to the trapping agent (Chou & Lipmann, 1952). Although added hydrogen carriers are not required when the crude fraction 1 is assayed (Seaman, 1953), fractions 2 and 3 require the electron acceptor system; lactic dehydrogenase and DPN were thus routinely included. The assay was carried out under nitrogen, and activity is expressed as μmole hydroxamic acid formed/hr./10 mg. protein.

In the *acetyl phosphate assay* (ACP assay), the non-enzymic acyl acceptor was replaced by 10 units of transacetylase, and 100 μmole phosphate buffer (pH 7·4) was used in place of Tris buffer. Added lactic dehydrogenase was also omitted from this assay since the bacterial extract used as the source of transacetylase contained sufficient lactic enzyme. Incubations were carried out under nitrogen for 60 min. at 32°. Acetyl phosphate formation was measured at the completion of the run and activity is expressed as μmole acetyl phosphate formed/hr./10 mg. protein.

Analytical methods. Acetyl phosphate and hydroxamic acid were measured as described by Lipmann & Tuttle (1945*a*, *b*). Protein content of enzymes was measured by ultraviolet absorption (Kalckar, 1947). The thioctic acid content of enzymes was determined by the growth response of tetrahymena or by the manometric assay procedure of Gunsalus, Dolin & Struglia (1952).

Preparations. Lactic dehydrogenase was crystallized from pig heart (Meister, 1952) and was assayed according to Mehler, Kornberg, Grisolia & Ochoa (1948). Transacetylase was supplied by a dialysed extract (Korkes, Stern, Gunsalus & Ochoa, 1950) of *Streptococcus faecalis* strain 10 C1. The streptococci were grown in defined medium low in thioctic acid (Gunsalus *et al.* 1952); the method of Stadtman (1952) was used to assay the preparations. Sodium pyruvate was prepared by the method of Robertson (1942). Cocarboxylase, DPN, and Co A were obtained commercially. Cultures of *S. faecalis* were kindly supplied by Dr I. C. Gunsalus, and thioctic acid was generously furnished by Dr E. L. R. Stokstad.

RESULTS AND DISCUSSION

Table 1 shows that the tetrahymena oxidase at the degree of purity of fraction 1 readily oxidized pyruvate in the ferricyanide system and formed acetyl-Co A as indicated by both the hydroxylamine trapping assay and by the formation of acetyl phosphate. Since in the hydroxamic acid assay, the incubation mixture contained both α-keto acid and hydroxylamine, which rapidly react to form the oxime, it appears that contrary to the condition in mammalian tissues, the protozoan oxidase utilizes the oxime derivative as readily as it does the α-keto acid. But fraction 1, when repeatedly frozen and thawed to yield fraction 2, yielded preparations which had only slight activity in the non-enzymic acyl acceptor assay system; activity of fraction 2 remained high in the ferricyanide and acetyl phosphate assays (Table 1). It thus appears that rather than being capable of utilizing oximes *per se*, the crude tetrahymena preparation (fraction 1) enzymically split the oxime, making both pyruvate and hydroxylamine available. The oxime-splitting system was in the material which was removed by the freezing and thawing procedure used in the preparation of fraction 2. Addition of material precipitated in this step to fraction 2, however, did not restore ability to react; the previously active protein was apparently denatured.

Table 1. *Activity of pyruvic oxidase fractions of different purity and the effect of alumina treatment.*

Conditions are described in text.

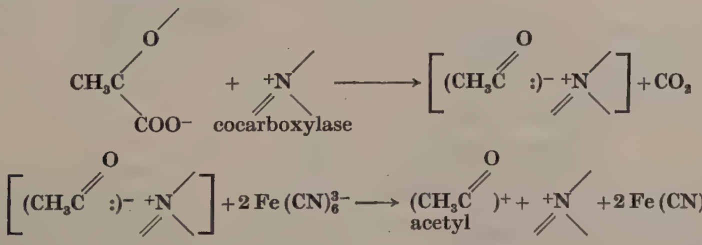
Enzyme fraction and treatment	Thioctic acid content of enzyme (mμg./mg. protein)	Pyruvic oxidase activity		
		FCNM assay	HXMA assay	ACP assay
Fraction 1				
None	37	3·8	1·8	1·1
Alumina	16	3·9	0·8	0·4
Alumina + 7·5 mμg. thioctic acid	16	3·6	1·5	0·8
Fraction 2				
None	96	10·4	0·4	3·2
Alumina	50	10·1	—	1·5
Alumina + 21 mμg. thioctic acid	50	9·4	—	2·7
Fraction 3				
None	139	18·3	—	5·0
Alumina	137	19·1	—	5·0

Additional evidence of an oxime-splitting system in crude preparations was afforded by pyruvate analyses of incubation mixtures containing fraction 1. Before addition of fraction 1, the hydroxamic acid assay reaction mixture gave a negative test for pyruvate with phenylhydrazine; all available pyruvate had combined with hydroxylamine. Ten minutes after the addition of the enzyme, traces of pyruvate were detected, and subsequent analyses during the entire 60 min. incubation period continued to indicate traces of the α-keto acid. In mixtures containing samples of fraction 1 inactivated by heating at 65° for 20 min., no pyruvate was detected. Since only small amounts of the added pyruvate are measurable at any one time, the equilibrium of the reaction: pyruvic oxime $+ H_2O \rightleftharpoons$ pyruvate + hydroxylamine, is well to the left. In the presence of active pyruvic oxidase the equilibrium may be shifted to the right as the α-keto acid is consumed and as hydroxylamine reacts with acyl groups. Oxime splitting must be slightly more rapid than pyruvate utilization since slight amounts of pyruvate were found throughout the incubation period. If the rate of the oxime system were slower, assays by the hydroxamic acid method would be lower than those obtained by the other procedures; Table 1 shows that this was not the case. According to theory, responses in the acetyl phosphate assay and in the hydroxamic acid assay should be equal, and about one-half that obtained in the ferricyanide assay. The observed lower response in the acetyl phosphate system resulted from competition between the *Streptococcus faecalis* extract and the tetrahymena enzyme for pyruvate. Although the streptococcal extract did not oxidize pyruvate via acetyl-Co A, significant amounts of acetylmethylcarbinol were formed from the α-keto acid.

With crude tetrahymena preparations one can therefore use hydroxylamine as the non-enzymic acceptor in the presence of α-keto acids. However, with purer preparations, in which the oxime-splitting system has been removed or destroyed (e.g. by repeated freezing and thawing), enzymic acceptor systems are needed in order to follow acyl-Co A formation.

Although alumina treatment (Seaman, 1953) of fractions 1 and 2 decreased the formation of acetyl-Co A, the treatment did not decrease the rate of pyruvate oxidation in the ferricyanide system. Assay of the fractions for thioctic acid demonstrated the actual decrease in concentration of co-factor after alumina treatment. The alumina procedure therefore, while not completely removing thioctic acid from the system, decreased the concentration of co-factor below enzyme saturation. Activity of the alumina-treated systems was readily restored by the addition of synthetic thioctic acid to incubation mixtures; 6–10 mμg. provided enough thioctic acid to reverse the alumina effect with fraction 1 (Fig. 1). With fraction 2, the optimal amount of thioctic acid was 20–28 mμg.

The findings that thioctic acid is not involved in the oxidation of pyruvate in the presence of ferricyanide is in agreement with previous reports (Seaman, 1953; Reed & DeBusk, 1953). The ferricyanide assay system gives an overall measure of the release of CO_2 involved in the oxidative decarboxylation of pyruvate (the decarboxylase and the initial oxidation of the two-carbon intermediate). This may be formulated as:



$$\underset{}{CH_3C(=O)COO^-} + \underset{\text{cocarboxylase}}{{}^+N} \longrightarrow \left[(CH_3\overset{O}{\overset{\|}{C}}\ :)^-\ {}^+N\right] + CO_2$$

$$\left[(CH_3\overset{O}{\overset{\|}{C}}\ :)^-\ {}^+N\right] + 2\,Fe(CN)_6^{3-} \longrightarrow \underset{\text{acetyl}}{(CH_3\overset{O}{\overset{\|}{C}}\ )^+} + {}^+N + 2\,Fe(CN)_6^{4-}$$

That ferricyanide is actually reduced in the assay mixture is indicated in Table 2. In these experiments, the reactants of the standard ferricyanide assay mixture, with the exception of the ferricyanide, were added to cuvettes

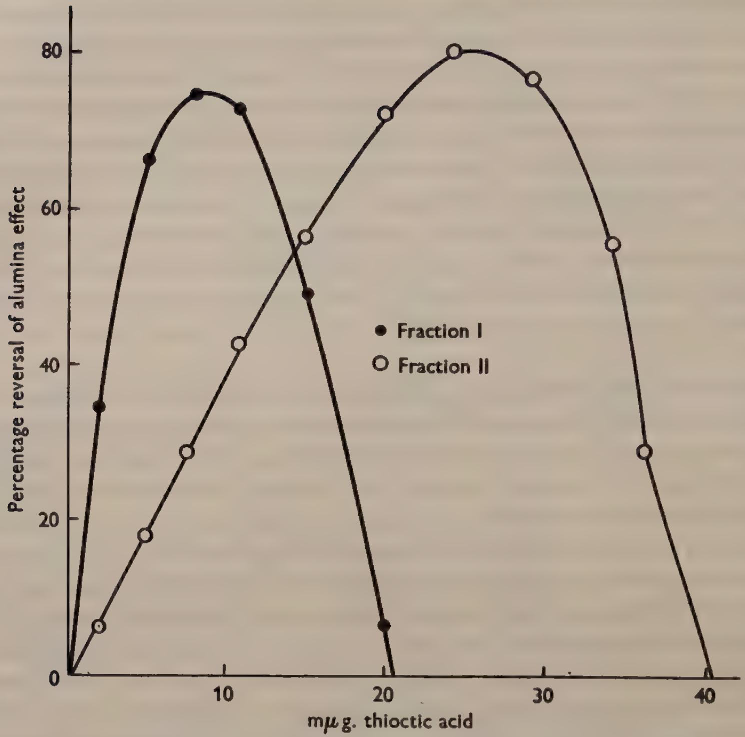


Fig. 1. Reversal of alumina effect by added thioctic acid. Activity measured in the ACP assay as described in text.

Table 2. *Ferricyanide reduction by pyruvic oxidase—Fraction* 1 *from tetrahymena*

Complete incubation mixture contained in 1·0 ml.: 33 μmole sodium pyruvate, 10 μmole $NaHCO_3$, 6·5 μmole $MgCl_2$, 72 μg. neutralized cocarboxylase, and enzyme. After incubation at 24 ± 1·5° for 5 min. the reaction was started by the addition of 0·5 μmole $K_3Fe(CN)_6$. Reduction was followed at wavelength 410 mμ. in the Beckman DU spectrophotometer.

Incubation mixture	Ferricyanide reduction μmole × 10^3/min./10 mg. protein
Untreated enzyme	
Complete	42
+0·1 μmole DPN	41
+7·5 mμg. thioctic acid	38
+5 units Co A	40
+5 units Co A+10 μmole cysteine	42
−pyruvate	2
−cocarboxylase	8
Alumina-treated enzyme	
Complete	40
+7·5 mμg. thioctic acid	36

and pre-incubated at room temperature (24 ± 1·5°) for 5 min. The reaction was then started by the addition of ferricyanide and the rate of reduction was followed in the Beckman DU spectrophotometer. As is the case with the pigeon enzyme system, the addition of Co A, DPN, or thioctic acid were without effect. In some experiments with Co A, cysteine was added to the reaction mixture to keep the coenzyme in the reduced form; this had no effect on the rate of reduction of ferricyanide. However, when the enzyme was omitted, there was, as expected, a rapid reduction of ferricyanide by the cysteine. It thus appeared that during the pre-incubation period of the assay, cysteine reacted with disulphide links in the enzyme protein and thus, as cystine, became unavailable to reduce ferricyanide. The reduced thiol groups of the protein would then maintain Co A in the reduced form, but could not reduce ferricyanide. This was verified by incubating heat-inactivated samples of fraction 2 (60° for 15 min.) with 0·0063 M-cysteine for 5 min. and then dialysing anaerobically against running nitrogen-saturated water for 48 hr. The resulting protein solution replaced cysteine in both the arsenolytic reaction with *Streptococcus faecalis* extracts, and in the acetate-activating reaction with crude pigeon liver extracts, but did not reduce an aqueous solution of ferricyanide.

The thioctic acid independence of the initial stage of pyruvate oxidation as formulated above appears to preclude the formation of an addition product of thioctic acid and pyruvate as an early step in oxidative decarboxylation; such a compound has been proposed by Calvin (1953).

Table 1 also shows that while the alumina treatment removed thioctic acid from fractions 1 and 2, with fraction 3 the procedure did not affect the rate of pyruvate oxidation or the thioctic acid content of the enzyme. This indicated that some component of the system which was required for the alumina effect, and which was present in crude material, was removed in preparing fraction 3. This is probably analogous to the removal during the purification of pigeon liver enzymes of an enzyme which split thioctic acid from enzyme protein (Seaman, 1954). The liberated thioctic acid was then adsorbed and removed by the alumina. However, unlike pigeon extracts, addition of the two subfractions obtained during this step to the resultant fraction 3, either singly or together, failed to restore ability of the fraction to show an alumina effect.

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The Distribution of Diaminopimelic Acid Decarboxylase among some Organisms of the coli-aerogenes Group and certain other Bacteria

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SUMMARY: Diaminopimelic acid decarboxylase was found to be widely distributed among 58 coli-aerogenes organisms. Different strains grown and tested under standard conditions showed great variations in enzyme activity although the amount of diaminopimelic acid in the cell hydrolysates was similar. Except for a higher average activity in *Aerobacter aerogenes*, there was no correlation between the activity of diaminopimelic acid decarboxylase and the biochemical type of coliform organism. No strain was found that contained diaminopimelic acid decarboxylase in the absence of lysine decarboxylase. The enzyme was also found in a strain of *Pseudomonas aeruginosa* but not in *Proteus vulgaris, Streptococcus faecalis* or *Bacillus subtilis.*

Acetone-dried preparations of certain strains of *Escherichia coli* have been found to contain an enzyme which decarboxylates natural α-ϵ-diaminopimelic acid to CO_2 and lysine (Dewey & Work, 1952*a*). The amount of this enzyme (diaminopimelic acid decarboxylase) varied from one strain to another and in three strains no activity was detected. Paper chromatographic analysis of the amino acids in acid hydrolysed cells showed that all the strains examined had approximately the same amount of diaminopimelic acid (Work & Dewey, 1953). Thus there is no obvious correlation between the degree of enzyme activity and the amount of substrate in the cell. The enzyme, with a slightly lower pH optimum, has also been found in a strain of *Aerobacter aerogenes* (Dewey & Work, 1952*b*).

The following survey of the distribution of diaminopimelic acid decarboxylase was undertaken to examine the distribution of the enzyme and to find whether this distribution could be correlated with any other characteristic.

METHODS

Organisms examined. The strains used were obtained from the Wellcome Bacterial Collection, the M.R.C. Microbiology unit at Cambridge, the National Collection of Type Cultures (NCTC) and the collection kept at this medical school. All strains were tested and classified according to their biochemical reactions, using the system described in *The Bacteriological Examination of Water Supplies* (1939; see Proom & Woiwod, 1951).

The culture medium used consisted of: Lab-Lemco (Oxoid) 10 g.; proteose peptone (Difco) 10 g.; NaCl 5 g.; glucose 20 g.; distilled water to 1 l. and adjusted to pH 7: 1 % (v/v) sterile blood was added when used for growing the streptococcus. The cultures were grown in Roux bottles containing 160 ml.

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medium; they were mechanically inoculated (Dewey & Wallace, 1953) and incubated at 37° for 12·5 hr. (the growth time for optimal enzyme activity in *Escherichia coli*; Dewey, Hoare & Work, 1954). After incubation the cells were harvested by centrifugation, washed once with water and mixed to a thin paste in water. About 10 vol. cold acetone was added to the paste with rapid stirring and the mixture left at 2° for 30 min. The cells were filtered on a Buchner funnel, washed with cold acetone and ether, and dried in vacuum.

Substrate. The diaminopimelic acid used had been isolated from hydrolysed cells of *Corynebacterium diphtheriae* (Work, 1951).

Estimations. The enzyme estimations were made by measuring the CO_2 evolved from diaminopimelic acid in a Warburg manometer at 38° with air as gas phase. Acetone dried cells (40 mg.) suspended in 2 ml. 0·1 M-phosphate buffer (pH 6·8) were placed in the main compartment and 0·5 ml. 0·025 M-diaminopimelic acid, dissolved in the same buffer, in the side bulb. The enzyme activity was measured as $Q_{CO_2} = \mu l.$ CO_2 released into the gas phase at pH 6·8/hr./mg. acetone-dried cells. No correction for CO_2 retention in the buffer was made in the rate determinations. When strains contained an active decarboxylase, 0·2 ml. 8 N-H_2SO_4 was tipped in from a second side bulb at the end of the reaction and the total gas change measured. Where the enzyme activity was low and the reaction not near completion after 1·5 hr. the enzyme action was stopped by acid tip and the products examined by paper chromatography.

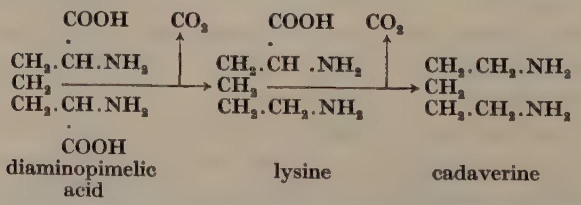
Chromatography. For chromatographic examination of reaction products the contents of Warburg flasks were neutralized with saturated $Ba(OH)_2$ and centrifuged; 40 $\mu l.$ of the supernatant fluid was chromatographed on Whatman No. 4 filter paper, with diaminopimelic acid, lysine and cadaverine as markers. Phenol (NH_3 atmosphere) was the solvent and the spots were developed with ninhydrin.

Amount of diaminopimelic acid in the cells. The approximate diaminopimelic acid content of the organisms was judged from the colour intensities of ninhydrin spots on two-dimensional paper chromatograms of electrodialysed, acid-hydrolysed cells (Work & Dewey, 1953).

RESULTS

Lysine decarboxylase

The lysine formed by the decarboxylation of diaminopimelic acid was itself decarboxylated to cadaverine and CO_2 by lysine decarboxylase also present in the acetone-dried cells.



Thus in the overall reaction two molecules of CO_2 were produced for each molecule of diaminopimelic acid added. In all the cells with a high enzyme activity 80–90 % of this theoretical CO_2 was produced. The paper chromatograms also showed that where decarboxylation had taken place the main product was always cadaverine; no appreciable amount of lysine was found, indicating that lysine decarboxylase was the more active enzyme and that diaminopimelic acid decarboxylase was the rate controlling enzyme.

Diaminopimelic acid decarboxylase

The distribution of diaminopimelic acid decarboxylase among the organisms tested is summarized in Table 1. Many of the strains showed a small gas change (5 μl.) when left in the Warburg apparatus for several hours, but no lysine or cadaverine was detected when the deproteinized vessel contents were examined by paper chromatography. All strains with a CO_2 evolution of less than 12 μl./hr. (Q_{CO_2} less than 0·3) are recorded as negative.

Table 1. *Distribution of diaminopimelic acid decarboxylase among organisms of the coli-aerogenes group*

		No. of strains with Q_{CO_2}			
	Total no. of strains	Less than 0·3	Between 0·3 and 2·0	Greater than 2·8	Presence of DAP in cell hydrolysate
Escherichia coli type 1	17	4	5	8	+
Escherichia coli type 2	2	1	1	0	nt
intermediate type 1	12	2	8	2	nt
intermediate type 2	4	0	2	2	nt
irregular	2	0	0	2	nt
Aerobacter aerogenes type 1	19	3	3	13	+
Klebsiella pneumoniae	2	0	1	1	+
	58				

DAP = diaminopimelic acid; nt = not tested.

The activity of diaminopimelic acid decarboxylase in *Aerobacter aerogenes* was generally higher than in other organisms examined (Fig. 1). There were, for example, 12 strains of *A. aerogenes* with Q_{CO_2} values greater than 5 as compared with 3 of the strains of *Escherichia coli* type 1; the average Q_{CO_2} for all the *Aerobacter aerogenes* strains tested was 6·25 as compared with 2·75 for *Escherichia coli* type 1. The organism with the highest activity was a strain of *Klebsiella pneumoniae* with $Q_{CO_2} = 24$ but the yield of cells was very small, being 130 mg. acetone-dried cells/l. medium compared with 500 mg./l. for the next most active organism, a strain of *Aerobacter aerogenes* with $Q_{CO_2} = 22$. A trace of enzyme activity was also found in a strain of *Pseudomonas aeruginosa* ($Q_{CO_2} = 1·0$) but no activity could be detected in a strain of *Proteus vulgaris* or one of *Bacillus subtilis*.

Diaminopimelic acid content of the cells

No difference could be detected in the colour strengths of the diaminopimelic acid spots in chromatograms from hydrolysates of seven strains of *Escherichia coli*, whereas the diaminopimelic acid decarboxylase activity among these seven strains varied from Q_{CO} values of 0 to 10. No irregular or intermediate types of *E. coli* were examined for the presence of diaminopimelic acid but the amount of this amino acid in hydrolysed *Aerobacter aerogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus subtilis* was almost the same as in *E. coli* type 1; *Streptococcus faecalis* contained neither diaminopimelic acid nor its decarboxylase.

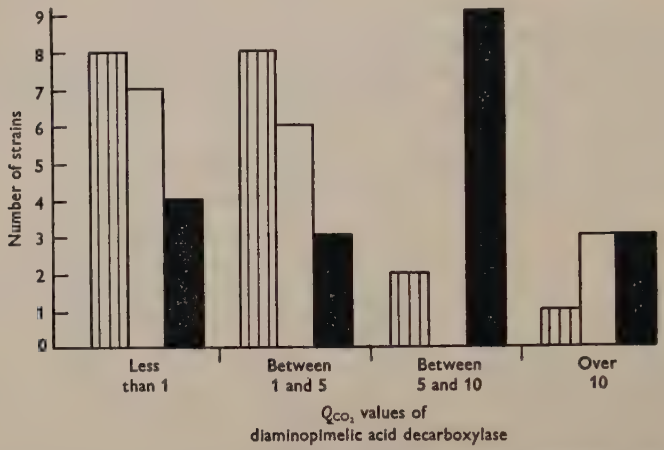


Fig. 1. The distribution of diaminopimelic acid decarboxylase activity: *Escherichia coli* types 1 and 2, striped columns; intermediate types, white columns; *Aerobacter aerogenes* type 1, shaded columns.

DISCUSSION

In this survey enzyme activity was estimated in organisms grown and tested under standard conditions. It was impracticable to find the effect of growth time and temperature on the enzyme activity of each organism, nor could the enzyme always be tested at its optimal pH. The phase of growth at harvesting has been found to be very critical with one strain of *Escherichia coli* but not with *Aerobacter aerogenes* (Dewey *et al.* 1954), while the pH optima for the enzyme from the two organisms differed by 0·4 units. The pH value 6·8 was chosen as a compromise between the optimum for the diaminopimelic acid and lysine decarboxylases, and the CO_2 retention by the buffer. The Q_{CO_2} values are thus not a true measure of diaminopimelic acid decarboxylase activity but are only relative and can only be compared when all the cells are tested at the same pH. The Warburg manometer was not sufficiently sensitive to detect very small amounts of enzyme; from the large number of strains found to contain only a trace, it seems probable that many of the strains recorded as 'negative' may contain traces of diaminopimelic acid decarboxylase below the limit of sensitivity of the test.

Cell-free extracts containing diaminopimelic acid decarboxylase are activated by pyridoxal phosphate (Dewey *et al.* 1954). As the complete acetone-dried cells were present in the Warburg cups, no additional pyridoxal phosphate was added. It is unlikely that the absence of enzyme activity could have been due to lack of co-enzyme rather than lack of the enzyme itself, since the organisms used are known to synthesize their own pyridoxal requirements.

There was no correlation between amount of enzyme and the amount of diaminopimelic acid in the corresponding cell hydrolysates. Diaminopimelic acid has been found in several organisms in which no decarboxylase could be detected, and two Gram-positive cocci which contained no diaminopimelic acid have been shown (Dewey, Hoare & Work, 1953) to contain diaminopimelic acid decarboxylase.

Davis (1952) suggested that diaminopimelic acid is an intermediate in the synthesis of lysine in *Escherichia coli* ATCC 9637. It seems unlikely that the strains which contain little or no diaminopimelic acid decarboxylase can derive all their lysine requirements from diaminopimelic acid. Roberts *et al.* (1953) reported that in another strain of *E. coli*, lysine was formed by a pathway not involving diaminopimelic acid. Evidence that diaminopimelic acid decarboxylase is active in normal cells of *E. coli* ATCC 9637 is shown by the fact that diaminopimelic acid accumulates in the culture medium of certain mutants which require lysine but which lack diaminopimelic acid decarboxylase (Dewey & Work, 1952*a*; Davis, 1952). The presence of an enzyme in acetone-dried cells is no proof that the enzyme is actually used by the growing cell, and further work is required before a metabolic role may be assigned to diaminopimelic acid decarboxylase.

Of 58 organisms of the coli-aerogenes group tested, 48 (83 %) contained detectable diaminopimelic acid decarboxylase activity. Gale (1946) reported that out of 151 strains of *Escherichia coli* tested 94 % contained lysine decarboxylase. That no strain has yet been found to contain diaminopimelic acid decarboxylase but not lysine decarboxylase seems probably due to the wide distribution of the latter enzyme in these organisms.

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The Inactivation of Free Bacteriophage by Irradiation and by Chemical Agents

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SUMMARY: Shigella phage S 13 and coli phage T 3 are very sensitive, in the free state, to inactivation by reducing radicals produced in water by ionizing radiation. They are inactivated by ascorbic acid, and by hydrogen peroxide (H_2O_2) acting as a reductant; but the H_2O_2 must decompose into active radicals before it attacks the phage. Hydrogen peroxide decomposes under irradiation, and attacks phage much more vigorously under ionizing radiation than under ultraviolet irradiation. Free radicals may part-inactivate phage, that is, render it more sensitive to H_2O_2 and to ascorbic acid, though not to inactivating agents in general. Phage grown on rich medium is more sensitive than phage grown on poor medium to inactivation by reduction.

Some results of general interest in microbiology have emerged from a series of investigations which were undertaken primarily to obtain information on the mode of action of ionizing radiations on bacteriophage. The main interest centred on 'indirect action', that is, the inactivating effects of the decomposition products of irradiated water. Interpretation of the radiation results necessitated a study of the effects of hydrogen peroxide and other inactivating agents on free phage. Certain conclusions have been reached as to the mechanism of inactivation by chemical agents, as well as by irradiation, when the phage is exposed in dilute suspension before adsorption to the bacterial host.

MATERIAL AND METHODS

Strains of bacteriophage and bacteria. Most of the experiments were done with the shigella phage S 13, active against *Shigella flexneri* strain Y 6 R; the stock was derived from that used by Lea & Salaman (1946). For plaque counts a sensitive *Escherichia coli* strain was used, as it was preferable to work with a non-pathogenic organism. When 'part-inactivation' of phage S 13 was discovered (Alper 1952*a*, 1954), experiments were done with the coli phage T 3 to see whether or not the phenomenon was general.

Preparation of bacteriophage stocks. For radiation experiments on dilute suspensions, bacteriophage stocks of high titre and low solid content were needed. To this end the phage was propagated on the surface of a solid medium of low nutrient content, made up of 0·5 % (w/v) Evans peptone and 2 % (w/v) agar. Petri dishes containing this medium were inoculated first with host cells derived from a single colony, and, after about 2 hr. incubation, with a phage suspension of suitable titre. After lysis of the host cells by the phage, the plates were each washed with about 2 ml. 0·001 M-phosphate buffer (pH 7·0) and the washings centrifuged at 6000 r.p.m. The water-clear supernatant usually had a phage titre of from 5.10^9 to 10^{11} particles/ml., and solid content between 2 and 10 mg./ml. In most of the experiments, suspensions were used at dilutions of about 10^{-6}.

The nature of the medium on which the phage was propagated was found to have a bearing on its sensitivity to radiation-produced active radicals and to hydrogen peroxide. For experiments specifically designed to examine this dependence, phage stocks were grown from the same host and phage clones both on peptone agar and on nutrient agar plates. The two types of S13 stock so derived will be referred to as S13P and S13R respectively.

Preparation and handling of dilute phage suspensions. To obtain clear-cut and reproducible results on dilute phage suspensions it was necessary to exclude as far as possible traces of organic or inorganic contaminating material. The chemicals used were always of analytical reagent quality; the water used in making the dilute suspensions (about 10^{-6}), the buffer and the other solutions, was twice distilled in glass from alkaline permanganate; glassware was stored in sulphuric + chromic acid cleaning solution, and very thoroughly washed before use, being given three final rinses with glass-distilled water. It was found desirable to eliminate the possibility that volatile substances from rubber teats might be absorbed in the dilute phage suspensions and influence their behaviour. Dropping pipettes were therefore made with ground glass cone terminals and fitted to joints specially made to attach to 1 ml. Luer-lock all-glass syringes.

Plaque counting: standard errors. Measured volumes of phage and bacterial suspensions were delivered by calibrated dropping pipettes on to nutrient agar plates and spread together over the surface. The plates were incubated for about 8 hr. at 37°, and the plaques then counted. The error of any plaque count included variations in drop size and ordinary statistical fluctuations. By making counts on 25 or more replicates, the standard deviation of any count n was found by experiment, and confirmed by periodic checks, to be $\sqrt{2n}$, instead of the theoretical $\sqrt{n}$. Thus the standard error of an observation of m replicates, with a mean count of n plaques per replicate, was $\sqrt{(2n/m)}$. Standard errors indicated in the diagrams have been calculated on this basis.

Irradiations. Neutrons and electrons from a van de Graaff generator, gamma-rays, and 200 kV. X-rays were used to investigate the effects of ionizing radiation. A Hanovia 'Germicidal' lamp, with most of the energy at 2537 A, was used as the source of ultraviolet light. Details will be published elsewhere concerning methods of irradiation and dosimetry, and of bubbling gases through phage suspensions undergoing irradiation.

Estimation of hydrogen peroxide. Interpretation of the radiation data depended on a knowledge of the amount of H_2O_2 formed by a given dose of ionizing radiation. Two methods were used: (1) for concentrations above 1 μg./ml., the method of Eisenberg (1943), in which titanous sulphate is oxidized to the titanic form and then to peroxide; (2) for lower concentrations, the method of Savage (1951), in which iodide is oxidized to iodine in the presence of starch, a coloured complex being formed.

RESULTS

Effects of hydrogen peroxide

When coli phage T3 or shigella phage S13 were exposed to H_2O_2, the curves of survival with time were usually of exponential form. The slopes of the curves depended on the conditions of the experiment, such as hydrogen peroxide concentration, temperature, and organic content of the phage suspension (Alper 1952*b*). Several other variables have now been investigated.

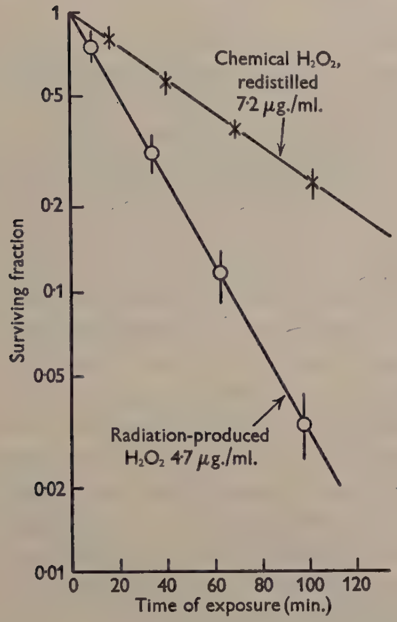

Fig. 1. Effect on coli phage T3 of radiation-produced and commercial H_2O_2.

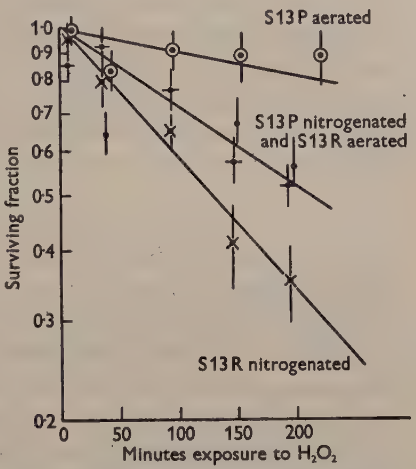

Fig. 2. Effect on phage S13P (poor medium) and S13R (rich medium) of H_2O_2; under air or nitrogen bubbling.

Source of hydrogen peroxide. With hydrogen peroxide formed by irradiation in clean aerated water or phosphate buffer, the effect on phage was almost always greater than that of an equal concentration of a commercial product, even of analytical reagent quality several times distilled. An example is illustrated by Fig. 1. The stabilizer contained in commercial hydrogen peroxide apparently inhibited the action of H_2O_2 on the phage. Repeated distillation did not entirely remove this stabilizer.

Method of phage stock preparation. Phage S13 when propagated on Evans peptone (phage S13P) was found to be less sensitive to H_2O_2 than when grown on nutrient agar (phage S13R) (Fig. 2).

State of aeration of suspension. Hydogen peroxide acted more effectively on phage suspensions from which oxygen was removed by bubbling nitrogen through the suspensions (Fig. 2).

Effects of other chemical agents

Ascorbic acid. Ascorbic acid was an inactivating agent for phage, in concentrations somewhat lower than those of H_2O_2 which produced the same effect. Survival curves were of exponential form. It was more active in deaerated suspensions, and more active against phage S13R than against phage S13P. Dehydroascorbic acid was inactive at comparable concentrations.

Chlorate and iodate. $KClO_3$ and KIO_3 were completely inactive against phage in solutions up to 10^{-3}M, i.e. in molar concentrations which were considerably higher than those of H_2O_2 or ascorbic acid which inactivated the phage.

Urea. Urea has been reported as an agent for breaking hydrogen bonds (Steinhardt, 1938; Alexander & Stacey, 1952), and for 'splitting' DNA molecules into two (Stacey & Alexander, 1953). Its action on phage was therefore thought to be of interest. In low concentration urea was inactive; at 4M (the concentration used by Stacey & Alexander) it inactivated phage S13, the shape of the survival curve being of non-exponential form (see Fig. 4).

Part inactivation

It was found that, after X-irradiation of dilute suspensions of shigella phage S13 or coli phage T3, many of the survivors had suffered a change, although they were still able to adsorb to bacteria and form plaques when plated out immediately. The change manifested itself as a greatly increased susceptibility to the action of hydrogen peroxide or ascorbic acid, but not to heat and urea (Figs. 3, 4). Phage which has been changed in this way by ionizing radiation will be referred to as 'part-inactivated'. The phenomenon did not occur with the survivors of ultraviolet irradiation, nor with phage exposed in concentrated suspension to ionizing radiation, when the inactivating effect of the radiation was of the 'direct' type. It was therefore concluded that part inactivation was due to active radicals formed by the action of radiation on water.

Phage stocks propagated on rich and poor media

Parallel phage stocks grown on 0·5% (w/v) Evans peptone agar (S13P) and on nutrient agar (S13R) responded differently to some inactivating agents and identically to others. Ultraviolet inactivation and the direct action of X-rays were identical for phages S13P and S13R (Figs. 5, 6). On the other hand, the indirect effect of X-rays, i.e. inactivation by active radicals, was much greater with phage S13R than with S13P (Fig. 7). The latter effect was also found for stocks of coli phage T3 propagated on the different media. As mentioned above, S13R was more susceptible than S13P to the action of H_2O_2 and ascorbic acid (Fig. 2).

The influence of dissolved gases on radiation effects

Immediate effect of radiation. It has been shown above that radiation may affect suspended bacteriophage through the H_2O_2 formed in aerated aqueous suspensions, and by bringing about part-inactivation, the effects of which are

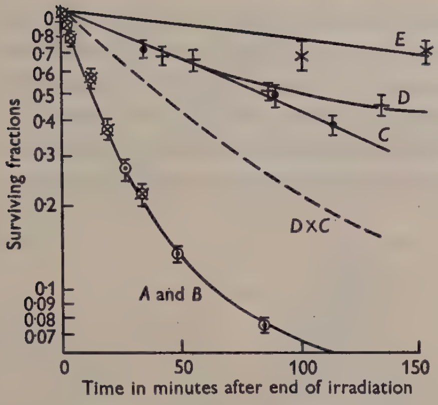

Fig. 3. Effect of irradiated buffer on irradiated and non-irradiated suspensions of shigella phage S13. ⊠*A*, irradiated suspension; ⊙*B*, irradiated phage diluted in irradiated buffer; ● *C*, non-irradiated phage diluted in irradiated buffer; +*D*, irradiated phage diluted in non-irradiated buffer; ×*E*, control.

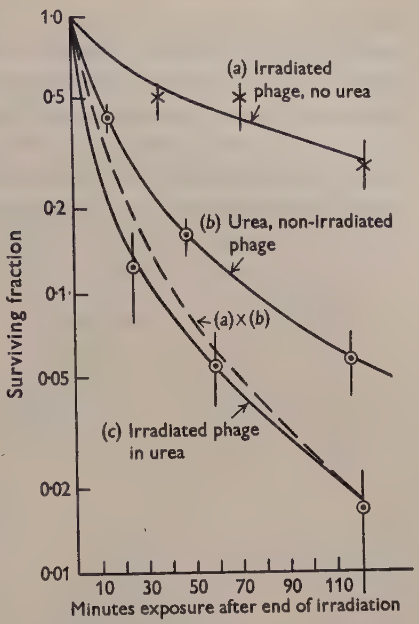

Fig. 4. Effect of urea on irradiated and non-irradiated suspensions of phage S13.

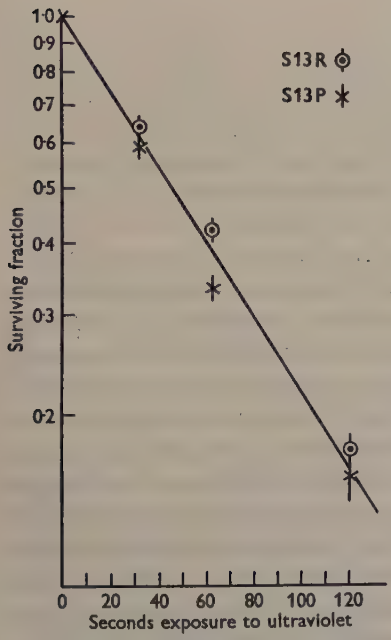

Fig. 5. Effect of ultraviolet radiation on phages S13P and S13R.

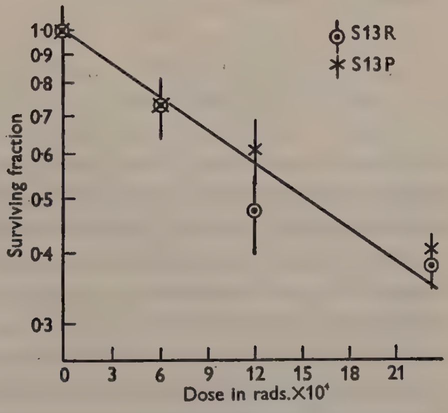

Fig. 6. Direct effect of X-rays on phages S13P and S13R.

persistent and may be followed after the end of the irradiation. Part-inactivation, and the formation of H_2O_2, occur as a result of reactions with and between the short-lived products of irradiation, generally agreed to be free radicals, which are present only while the irradiation is proceeding. These radicals may themselves inactivate the phage during irradiation, and this inactivation will be called the *immediate* effect of radiation. It has been found possible to investigate this alone by delivering radiation in a time which is short compared with that needed for the action of the formed H_2O_2, and by sampling the phage very soon after the end of an irradiation. The mechanisms of radical action on phage have to some extent been elucidated by studying the effectiveness of radiation delivered at a high dose rate under the influence of various gas treatments. It was found that the immediate effects of radiation were greatest where the gas treatment was such as to make the maximum number of reducing radicals available to the phage. Fig. 8 illustrates an experiment in which the survival of phage S13 was followed when it was irradiated in the presence of hydrogen, nitrogen, air, and a mixture of hydrogen and oxygen. The probable nature of the reducing and oxidizing radicals formed under these treatments has been discussed elsewhere (Ebert & Alper, 1954). The radiation was about ten times as effective under hydrogen bubbling, where reducing reactions were favoured, as under hydrogen+oxygen bubbling, where reducing radicals were rapidly removed and made unavailable to the phage.

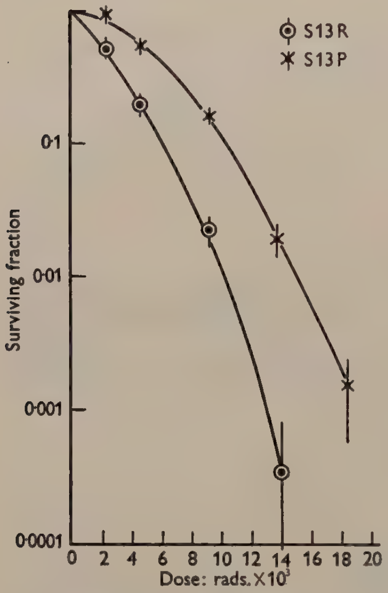

Fig. 7. Indirect effect of X-rays on phages S13P and S13R.

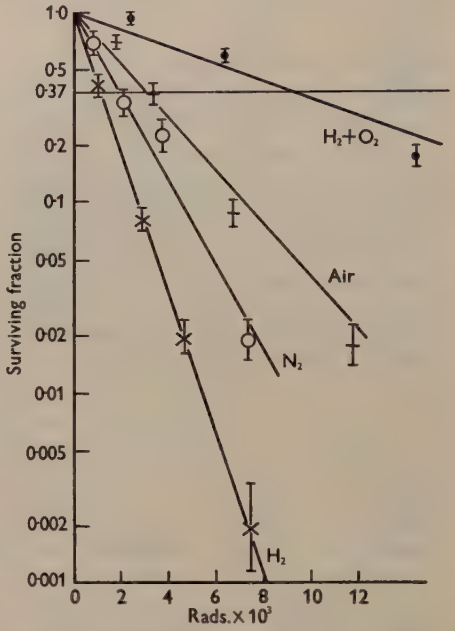

Fig. 8. Influence of various gas treatments on indirect inactivation by X-rays.

Part-inactivation. In aqueous suspensions from which oxygen has been removed, H_2O_2 is formed by radiation in negligible amounts (Bonet-Maury, 1952). The occurrence of part-inactivation in an air-free suspension would therefore not become manifest unless the survivors of the irradiation were exposed to H_2O_2 or ascorbic acid. Experiments were done to investigate the relative extent of part-inactivation occurring in aerated and air-free suspensions. Two phage suspensions and two equal volumes of buffer were irradiated, one of each under aeration, and the other air free. At the end of the irradiation, the air-free buffer was added to the irradiated aerated phage suspension, and the aerated buffer to the air-free phage suspension. Thus the survivors in both suspensions were exposed, after irradiation, to equal concentrations of H_2O_2. As can be seen from Fig. 9, the inactivation of the suspension which had been irradiated in the absence of oxygen was somewhat more rapid. Thus part-inactivation, as well as immediate inactivation, appears to arise from reaction with reducing radicals.

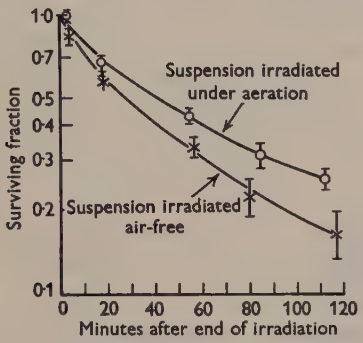

Fig. 9. Post irradiation effect of H_2O_2 on suspensions of phage S13 irradiated with and without aeration.

Effects of hydrogen peroxide under irradiation

When phage was introduced into previously irradiated buffer and then exposed to further ionizing radiation, the total effect was greater than the sum of the effects of radiation and peroxide acting independently. In a typical experiment phosphate buffer (pH 7·0; 0·001 M) was irradiated with 11,000 roentgens of γ-rays, a dose which formed about 1 μg. H_2O_2/ml. buffer. Phage was introduced into two ampoules (1) and (2) containing the irradiated buffer; ampoule (1) was then subjected to further γ irradiation. In addition, phage was irradiated by the same source of γ-rays without previous irradiation of the buffer (ampoule (3)). The three survival curves are presented together in Fig. 10. The effect of irradiating the phage in the previously irradiated buffer was considerably greater than the sum of the separate effects of irradiated buffer and irradiation, particularly in the earlier stages of irradiation, when no appreciable amount of H_2O_2 had yet been formed in ampoule (3). By contrast, the effect of ultraviolet light on phage in the presence of H_2O_2 was no greater than the sum of the separate effects of the H_2O_2 and the ultraviolet radiation.

DISCUSSION

The majority of biological radiation effects appear to be oxidative in nature (Gray, 1953) as judged mainly by the fact that the presence of oxygen enhances the effectiveness of the radiation. The radiation experiments with phage show that with this system reducing radicals are the inactivating agents, although the presence of oxygen during radiation might give rise to enhanced effects through the formation of hydrogen peroxide, if part-inactivation has taken place and if there is a time lapse between the radiation period and the time of sampling. That radiation reduction processes in other biological systems might be disguised as oxidations, because of oxygen enhancement, is suggested by work of Daniels, Scholes & Weiss (1953). These authors found that the viscosity of thymus deoxyribosenucleic acid was immediately affected by radiation to a greater extent in the absence of oxygen than in its presence; but the after-effects were considerably more when oxygen was present during irradiation.

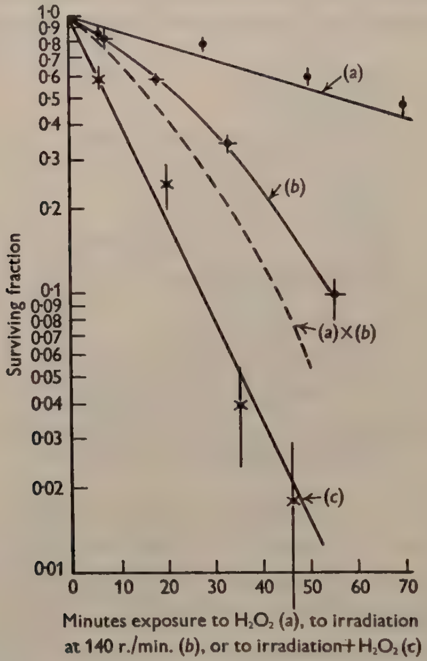

Fig. 10. Effect of H_2O_2 on phage S13 undergoing further irradiation.

The conclusion that free phage is very sensitive to reducing agents is supported by the inactivating effect of ascorbic acid and by the lack of effect of oxidizing agents in concentrations of much higher molarity. It is usually assumed that H_2O_2 acts on biological systems by virtue of its oxidizing properties, but it can also act as a reductant, as with strong oxidizing agents such as $KMnO_4$. That H_2O_2 acts as a reductant on bacteriophage is inferred from the experimental work with other reducing agents, and also from the fact that the effects of H_2O_2 on phage suspensions are enhanced when oxygen is removed by the passage of nitrogen through the suspension. From experiments in which H_2O_2 which had not been freed from stabilizer was used, it is inferred that before H_2O_2 is effective in inactivating phage it must decompose. H_2O_2 decomposition is brought about by radiation, and under γ-irradiation the effect of the H_2O_2 was much greater than without irradiation, allowing for inactivation due to the γ-irradiation. With decomposition of hydrogen peroxide by ultraviolet radiation, on the other hand, no enhanced effect on the phage was observed.

Under ultraviolet irradiation, the reaction: $H_2O_2 \rightarrow OH + OH$, takes place (Urey, Dawsey & Rice, 1929). The hydroxyl radicals may in their turn react with H_2O_2 molecules, producing free radicals of other types. The steps in H_2O_2 decomposition under ionizing radiation are not known, but from the present work it may be inferred that the proportion of reducing radicals made available during ionizing irradiation is very much higher than with ultraviolet irradiation. In experiments previously reported (Alper, 1952*b*) it was shown that when an aerated phage suspension was irradiated by γ-rays at a low dose rate in the presence of catalase, the shape and slope of the survival curve indicated that the catalase protected the phage by decomposing the formed H_2O_2. This protection could be given only if the catalase decomposed the H_2O_2 via harmless radicals, as in fact is likely in ordinary biological decomposition of H_2O_2, whereas without catalase present the H_2O_2 could attack the phage by decomposition through other reactions. The decomposition of H_2O_2 by catalase was considered by Weiss (1935) to proceed via OH radicals.

The reduction of phage particles was observed in those conditions in which reactions took place with inactivating agents arising from, or dissolved in, the suspending medium; this suggests the possibility of reactions at the surface of the particles. The phenomena associated with reducing processes (protective effect of oxygen, enhanced sensitivity to ascorbic acid) do not arise under the direct action of X-rays, nor with inactivation by ultraviolet radiation, with which there is no 'indirect effect' since, at the wave lengths used, the energy is not absorbed in the suspending medium. Watson (1952) suggested that the mechanism of inactivation by indirect action of X-rays might be different from that of direct action. He obtained some evidence that phage inactivated by indirect action failed to adsorb, whereas with ultraviolet or direct X-ray inactivation (Watson, 1951) adsorption is unimpaired. It seems likely that the inactivation of phage by reducing agents arises from an interference with one of the steps in adsorption to the bacterial host. (Puck, Garen & Cline, 1951; Garen & Puck, 1951; Tolmach & Puck, 1952). Experiments are planned which, it is hoped, will yield further information on this point.

The part-inactivation phenomenon, which itself occurs under irradiation as a result of reaction with reducing radicals, suggests that the reduction of a phage particle may take place in two steps, the first of which does not impair its ability to adsorb to bacteria but does increase the probability of a second reaction with a reducing agent. A phage population which has survived the immediate effects of a small dose of radiation is composed of part-inactivated particles and of those which have escaped any action of the free radicals. Initially the action of a post-irradiation inactivating agent proceeds rapidly, until only those particles are left which are inactivated at the same rate as those in non-irradiated suspensions. Post-irradiation survival curves can in fact be resolved into two exponential curves, the less steep one being parallel to the survival curve of non-irradiated phage exposed to the same agent (Fig. 11). It can be inferred that inactivation by reducing agents is a first order reaction, the probability of its occurrence at any collision being greatly increased with part-inactivated phage.

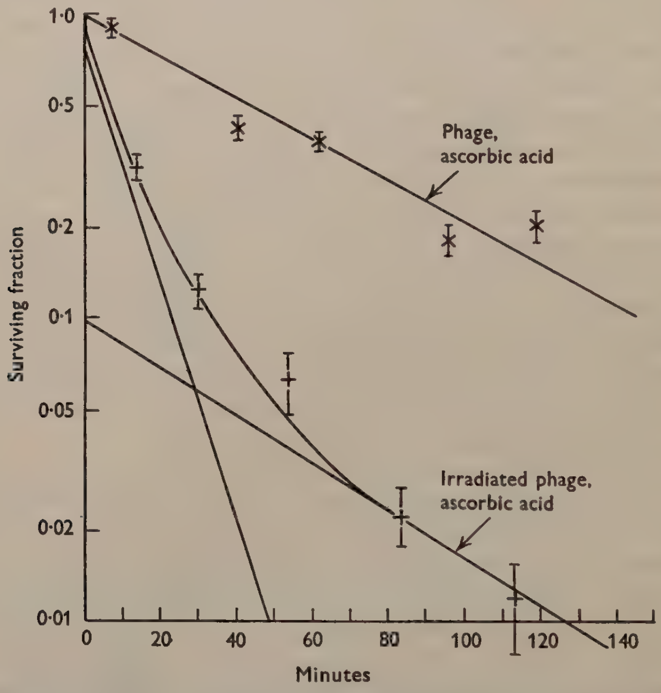

Fig. 11. Effect of ascorbic acid on irradiated and non-irradiated suspensions of phage S13.

Inactivation by agents such as ultraviolet radiation and the direct action of X-rays proceeds at the same rate for phage S13P (propagated on poor medium) as for phage S13R (rich medium). Inactivation by reducing agents, on the other hand, proceeds faster with S13R. Cohen (1947) described different inactivation rates of stocks of coli phage T2 grown on rich or poor media, and concluded that the difference arose from the fact that phage particles grown on poor media were coated with a layer of deoxyribosenucleic acid, probably of bacterial origin. Experiments have not yet been done to see whether the differences between S13P and S13R disappear after digestion with deoxyribonuclease. It is noteworthy, however, that by contrast with coli phage T2 grown by Cohen on poor media, S13P stocks have remained stable over many months. Moreover, whenever there were differences in the responses of phages S13P and S13R to inactivating agents, the former was more resistant, in contrast to Cohen's findings with coli phage T2. If these differences between S13P and S13R are in fact due to the presence of a deoxyribonucleic acid layer on the surface of S13P, it would follow that this layer protects against reduction and is itself sensitive to reducing agents.

I am greatly indebted to Dr M. Ebert for active collaboration in some of the experiments here described and for his unfailing assistance during much of the investigation. It is a pleasure to acknowledge the stimulating effects of discussions with Dr L. H. Gray and Dr William Hayes. Most of the work was done while I was in receipt of a personal research grant from the Medical Research Council.

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Some Properties of Influenza Virus Filaments shown by Electron Microscopic Particle Counts

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SUMMARY: A strain of influenza virus A which produced abundant filaments was more efficient in agglutinating red cells than non-filamentous strains. This was shown by counts of the number of particles (i.e. filaments and spheres)/agglutinating dose; the ratios found were $10^{6\cdot6}$ for the filamentous, and $10^{7\cdot2}$ for the non-filamentous strains. The increased efficiency of agglutination was shown by filtration experiments to be due to the filaments. There was no corresponding increase in the efficiency of initiating infection. Treatment of a filamentous preparation with ultrasonic vibrations caused breakdown of the filaments, an increase in agglutination titre but no change in the infectivity titre.

In a recent study of counts of influenza virus particles by electron microscopy it was found that for four strains of virus examined there was an average of $10^{7\cdot2}$ virus particles/agglutinating dose, as defined below (Donald & Isaacs, 1954). One strain gave a figure of $10^{6\cdot9}$ in two different experiments, however, and it was noticed that these two preparations contained a number of filamentous forms of virus. The possibility was considered, therefore, that virus filaments might show different agglutinating or infective behaviour from spherical forms. This possibility was investigated with a strain of influenza virus, A/Persian Gulf/2/52, known to produce many filamentous forms. This paper reports the results of our investigation.

MATERIALS AND METHODS

The techniques for counting and biological assay of influenza virus described in an earlier paper (Donald & Isaacs, 1954) were used with only a few modifications.

Technique of counting. Previously two techniques were employed—a spray technique and counts of the number of particles adsorbed on fowl red cell ghosts; these two techniques gave essentially the same results. The spray technique was unsuitable for the present experiments, however, since virus filaments were not found in sprayed materials. The red cell technique was therefore used alone. For purposes of counting each filament was counted as a single particle.

Agglutinating dose. An agglutinating dose (A.D.) is defined as the least amount of virus which causes partial (50%) agglutination of 0·25 ml. of a 1% suspension of chick red cells (approximately $10^{7\cdot15}$ cells) in a pattern test.

Infectivity titrations. Titrations were carried out in the allantoic cavity of the chick embryo as described previously and with 4 eggs/half-log dilution.

Ultrasonic treatment. For the 350 kc. treatment a quartz crystal oscillator built at the National Institute for Medical Research was used. Experiments were carried out at a power output of 15 W./cm.^{2} with an effective crystal area of 19 cm.^{2}. Volumes (3·0 ml.) of the allantoic fluids to be treated were contained in soda glass test tubes 19 cm. long and of 1 cm. internal diameter. Runs of 3 min. were carried out in ½ min. bursts, with immersion of the tubes at 0° between bursts, so that the temperature of the fluids under test at no time exceeded 30°.

A magnetostriction generator was used for the 6 kc. tests. Because of the low power output of this machine, fluids were contained in tubes with polythene bases.

RESULTS

The strain of virus used in these experiments produces large numbers of filamentous forms during its growth in the allantoic cavity of the chick embryo. The appearance on electron microscopy is illustrated in Pl. 1, fig. 1. Counts were made of the number of filaments + spheres in different preparations; the counts were related in each case to the agglutination titre of the corresponding preparation. The results of a number of experiments are shown in Table 1; they are expressed as the ratio of particles counted (i.e. particles = filaments + spheres)/agglutinating dose. For comparison, the results of counts of non-filamentous strains are shown.

Table 1. *Comparison of ratio of particles to an agglutinating dose in filamentous and non-filamentous strains of influenza virus*

Virus	Ratio ($\log_{10}$) of particles to an agglutinating dose
Filamentous strain (A/ Persian Gulf/2/52)	6·4, 6·7, 6·5, 6·6, 6·6, 6·6, 6·6, 6·7, 6·5, 6·6, 6·6, 6·5, 6·5, 6·6, 6·6, 6·6
Non-filamentous strains	7·3, 7·4, 7·2, 7·1, 7·3, 7·1, 7·3, 7·1, 6·9, 7·3, 7·3, 7·2, 7·3, 7·2, 7·2

Mean ratio for filamentous strain 6·6 ± 0·08
Mean ratio for non-filamentous strains 7·2 ± 0·13
Difference 0·6 ± 0·15

$t=15{\cdot}9$; $m=29$; $p<0{\cdot}001$

It is clear that A/Persian Gulf/2/52 gives a significantly lower ratio of particles/agglutinating dose than strains without filaments. These results show that agglutination is a more efficient process, i.e. fewer particles are required to cause a standard amount of agglutination in A/Persian Gulf/2/52 than in the other strains. By themselves, the results do not, however, allow us to conclude whether it is the filaments which are responsible for the increased efficiency of agglutination, rather than a strain peculiarity of another sort. To answer this question two types of experiment were undertaken; filtration was successful but centrifugation unsuccessful in separating filaments from elementary bodies.

Filtration experiments

Allantoic fluid from eggs infected with A/Persian Gulf/2/52 virus was filtered through collodion membranes of average pore diameter (A.P.D.) about 0·7 μ. Filtration usually resulted in a loss of about 75–90 % of the virus haemagglutinin and filtrates contained no long filamentous forms but only spheres and short rods (Pl. 1, fig. 2). Counts were made of number of particles/agglutinating dose in filtrates and the results compared with the same materials before filtration. The results are shown in Table 2. It is clear that the ratio for filtrates is significantly higher than the ratio for the unfiltered fluids. We conclude therefore that the increased efficiency of agglutination shown by this strain of virus is due to its filamentous forms.

Table 2. *Effect of filtration through a collodion membrane* (A.P.D. 0·7 μ) *on the ratio of particles to an agglutinating dose in A/Persian Gulf/2/52 virus*

Material examined	Ratio ($\log_{10}$) of particles to an agglutinating dose
Allantoic fluid	6·6, 6·5, 6·6, 6·5, 6·5, 6·6, 6·6, 6·6
Filtrate	7·0, 7·0, 6·9, 7·0, 7·0, 6·9, 7·1, 6·9

Mean ratio for allantoic fluid 6·6 ± 0·06
Mean ratio for filtrate 7·0 ± 0·07
Difference 0·4 ± 0·1
$t = 11{\cdot}4$; $n = 7$; $p = < 0{\cdot}001$

Centrifugation experiments

The filaments were readily sedimented by low-speed centrifugation, 1500 *g* for 15 min. The deposits after centrifugation always contained many spherical forms in addition to filaments; however, we do not know whether these spheres are carried down on larger particles or result from breaking of the filaments during centrifugation and re-suspension of the deposit. Nevertheless deposits usually contained a slightly higher proportion of filaments to spheres than the original fluids; in one experiment the proportion of filaments rose from 33–75 %. Electron micrographs of the supernatant and deposit obtained after centrifugation for 15 min. at 1500 *g* are shown in Pl. 2, figs. 3 and 4. Counts of the ratio of particles/agglutinating dose in the supernatants and deposits are shown in Table 3. The supernatants gave slightly higher ratios but the difference between the two is not statistically significant.

Table 3. *Effect of centrifugation at* 1500 *g on the ratio of particles to an agglutinating dose in A/Persian Gulf/2/52*

Material	Ratio ($\log_{10}$) of particles to an agglutinating dose				
Supernatant after centrifugation	7·1	7·0	6·7	6·6	6·7
Deposit after centrifugation	6·8	6·6	6·6	6·5	6·7

Mean ratio for supernatant 6·8 ± 0·2
Mean ratio for deposit 6·6 ± 0·1
Difference 0·2 ± 0·25
$t = 2{\cdot}4$; $n = 4$; $0{\cdot}1 > p > 0{\cdot}05$

Effect of ultrasonics on influenza virus filaments

It was of interest to see what effect breaking influenza virus filaments would have on the agglutination titre and on the ratio of particles to an agglutinating dose. Accordingly treatment with ultrasonics was carried out using 3-min. treatments at 6 kc. and 350 kc.

Treatment at 6 kc. produced a rise in the agglutination titre of A/Persian Gulf/2/52 virus of 2- to 4-fold. The deposit after centrifugation at 1500 *g* for 15 min. showed a similar rise in titre after ultrasonic treatment but filtrates through 0·7 μ. A.P.D. membranes showed no rise in titre. Treatment at 350 kc. produced a 2- to 6·5-fold rise in agglutinin titre with A/Persian Gulf/2/52 virus. Non-filamentous strains, e.g. P.R. 8 or N.W.S. or a filtrate of A/Persian Gulf/2/52 tested at the same time, showed no significant changes in agglutinin titre. Results of some representative experiments illustrating these points are shown in Table 4. The appearances found after treatment with ultrasonics are shown in Pl. 2, figs. 5 and 6. It will be seen that most of the larger filaments had broken up into rods and spheres. No significant changes in the ratio of particles to an agglutinating dose were found after treatment at 6 kc. At 350 kc. it appeared that quite a considerable rise in ratio occurred, but as can be seen from inspection of Pl. 2, fig. 6, it is often extremely difficult to decide which particles to count owing to the debris present. This question was therefore not investigated further.

Table 4. *Effect of ultrasonic vibrations on the haemagglutinin titre of filamentous and non-filamentous strains of influenza virus*

Expt.	Virus material tested	Frequency of ultrasonic vibration used (k.c.)	Haemagglutinin titre	
			Before treatment	After treatment
1	A/Persian Gulf/2/52	350	960	6400
	A/Persian Gulf/2/52 filtrate	350	240	360
2	A/Persian Gulf/2/52	350	3840	17,920
	N.W.S. (non-filamentous)	350	2560	3200
3	A/Persian Gulf/2/52 deposit	6	80	320
	A/Persian Gulf/2/52 filtrate	6	100	80
4	A/Persian Gulf/2/52	6	960	1920
	A/Persian Gulf/2/52 deposit	6	60	120
	A/Persian Gulf/2/52 filtrate	6	240	240

Filtrate: obtained after filtration through 0·7 μ. A.P.D. membrane.
Deposit: obtained after centrifugation for 15 minutes at 1500 *g*.

The relative infectivity of virus filaments and spherical forms

It was found on examining strains containing mainly spheres that the average ratio of particles to an agglutinating dose was $10^{7\cdot 2}$, the average ratio of infective doses (I.D. 50) to an agglutinating dose was $10^{6\cdot 1}$ and hence that roughly 10 virus particles corresponded to one I.D. 50 dose. Since filaments were more efficient agglutinating agents than spheres it was of interest to see whether they were also more efficient infective agents. It was surprising to find, however, that A/Persian Gulf/2/52 gave a significantly lower ratio of I.D. 50 to an agglutinating dose than did other strains we have examined, and that the ratio of particles to an I.D. 50 with this strain was again about 10.

In order to measure the contribution of the virus filaments to the infectivity of a preparation of virus it was necessary to be able to separate filaments from spheres efficiently. By filtration it has been possible to obtain spheres free of filaments but we have not been able to obtain a preparation of filaments free of spheres. The infectivity titres of preparations of freshly harvested allantoic fluid, filtrate and deposit after light centrifugation were measured and at the same time the agglutination titres, fraction of filaments to total particles expressed as a percentage, and total particle counts were noted. From these findings it was possible to calculate the ratio I.D. 50 doses/agglutinating dose, particles/agglutinating dose, and particles/I.D. 50 dose for preparations containing different proportions of filaments to elementary bodies. The results are shown in Table 5. The variations found in the ratios of particles/I.D. 50 dose for allantoic fluid, filtrate or deposit after centrifugation in different experiments, showed no consistent trends. The present techniques do not therefore show any differences in the relative infectivity of virus filaments and spheres.

Table 5. *Effect of filtration and centrifugation on infectivity of A/Persian Gulf/2/52 virus*

	Material														
	Allantoic fluid					Filtrate					Deposit				
Experiment:	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Ratio ($\log_{10}$) I.D. 50/A.D.:	4·9	5·8	5·6	5·4	5·3	5·5	5·5	5·9	5·5	5·6	4·8	5·6	5·9	5·3	5·2
Ratio ($\log_{10}$) particles/A.D.:	6·6	6·5	6·6	6·6	6·6	6·9	7·0	6·9	7·1	6·9	6·6	6·5	6·6	6·5	6·3
Ratio ($\log_{10}$) particles/I.D. 50:	1·7	0·7	1·0	1·2	1·3	1·4	1·5	1·0	1·6	1·3	1·8	0·9	0·7	1·2	1·1
Filaments (%):	40	60	40	50	33	—	—	—	—	—	50	70	60	64	75
Mean ratio ($\log_{10}$) I.D. 50/A.D.:	5·4					5·6					5·4				
Mean ratio ($\log_{10}$) particles/A.D.:	6·6					7·0					6·5				
Mean ratio ($\log_{10}$) particles/I.D. 50:	1·2					1·4					1·1				
Mean percentage filaments:	45					—					64				

Effect of ultrasonics on the infectivity of filamentous preparations of influenza virus

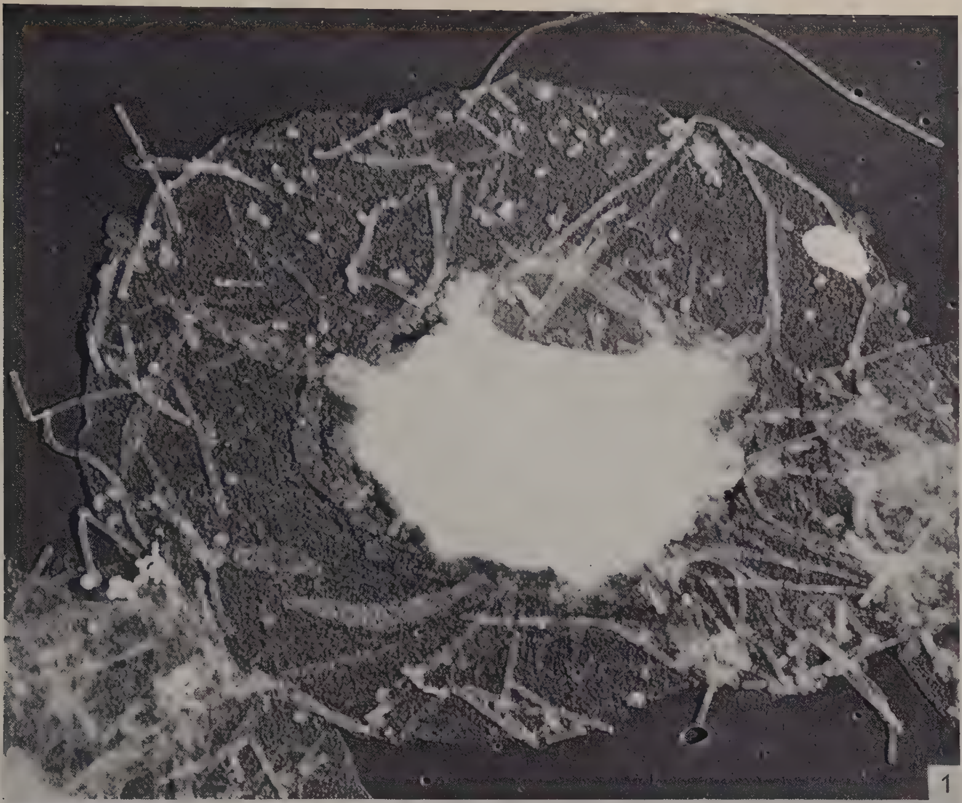
A number of experiments was carried out to test the effect of ultrasonic treatment at 6 kc. and 350 kc. on the infectivity of A/Persian Gulf/2/52 virus. Invariably there was no significant change in infectivity titre following treatment with ultrasonic vibrations. Thus in one experiment the haemagglutinin titre rose after treatment at 350 kc. from 1600 to 10,240 but the infectivity titre (titrated twice) remained unchanged. Ultrasonic treatment of a non-filamentous strain under similar conditions (350 kc.) produced no change in infectivity.

DISCUSSION

These experiments show that the ratio of particles to an agglutinating dose is about 0·6 log unit higher for a filamentous than for a non-filamentous strain of influenza virus. If we take into account the fact that the filamentous strains contained on the average about 50% filaments and 50% spherical forms we can conclude that a virus filament is, on the average, about 7 or 8 times more efficient than a sphere in producing agglutination in the pattern test. It is interesting to note therefore that it was possible to increase the agglutinin titre of a filamentous preparation up to 6·5 times by treatment with ultrasonic vibrations at 350 kc. Thus on ultrasonic treatment 20–40 agglutinating units, on the average, appear to have been produced for each filament; this figure is necessary to account for the decrease in the efficiency of agglutination and the rise in agglutinin titre which occurred.

Filamentous forms of influenza virus were first noted by Mosley & Wyckoff (1946). Chu, Dawson & Elford (1949) found that recently-isolated strains of influenza virus A frequently showed long filamentous forms and they demonstrated that the filaments possessed many of the properties of influenza virus elementary bodies. Hoyle (1950) made dark field microscopic observations which led him to conclude that protrusions of the allantoic cells occur in normal and in infected membranes and that the protrusions in the infected membranes contain virus material. Wyckoff (1951) examined by electron microscopy ultra-thin sections through cells which were apparently excreting influenza virus filaments; he found that the contents of the filaments were continuous with those of the cell cytoplasm. The work of Chu, Dawson & Elford (1949), Hoyle (1950), Murphy, Karzon & Bang (1950) and Wyckoff (1951) suggests that, during the growth of most strains of influenza virus A, filaments are formed first and later break down to give spherical forms, but that in many recently isolated strains, such as A/Persian Gulf/2/52, this last process is slowed down. The results of our experiments with ultrasonic treatment of a filamentous virus strain suggest that natural breakdown of filaments to spheres would cause a rise in haemagglutinin titre. A rise in haemagglutinin titre as a result of natural breakdown of filaments might be accompanied either by no change in infectivity titre or by a corresponding rise in infectivity. A rise in infectivity paralleling a rise in agglutination titre would mean that filaments and spheres would show about the same ratio of I.D. 50 doses to an agglutinating dose. So far as can be seen from our experiments filtrates of A/Persian Gulf/2/52 virus show a ratio of I.D. 50 doses to an agglutinating dose which is about equal to that of preparations of this strain containing abundant filaments. If the spheres are in fact normally derived from breakdown of the filaments, our results would suggest that a filament with a probability of about 1 in 10 of initiating infection in the allantoic cavity is naturally broken down into a number of spherical forms each with a probability of about 1 in 10. Hence a virus filament has a greater efficiency than a sphere in agglutinating red cells but this is unaccompanied by any increased efficiency in initiating infection.

In our experiments with ultrasonic vibrations of a filamentous strain the rise in agglutinin titre was unaccompanied by any change in infectivity. We might suppose that a rise in infectivity was masked by a corresponding drop due to virus inactivation, but there is no support for this suggestion from similar experiments with a non-filamentous strain in which no virus inactivation occurred. We are left with the belief that natural breakdown of filaments



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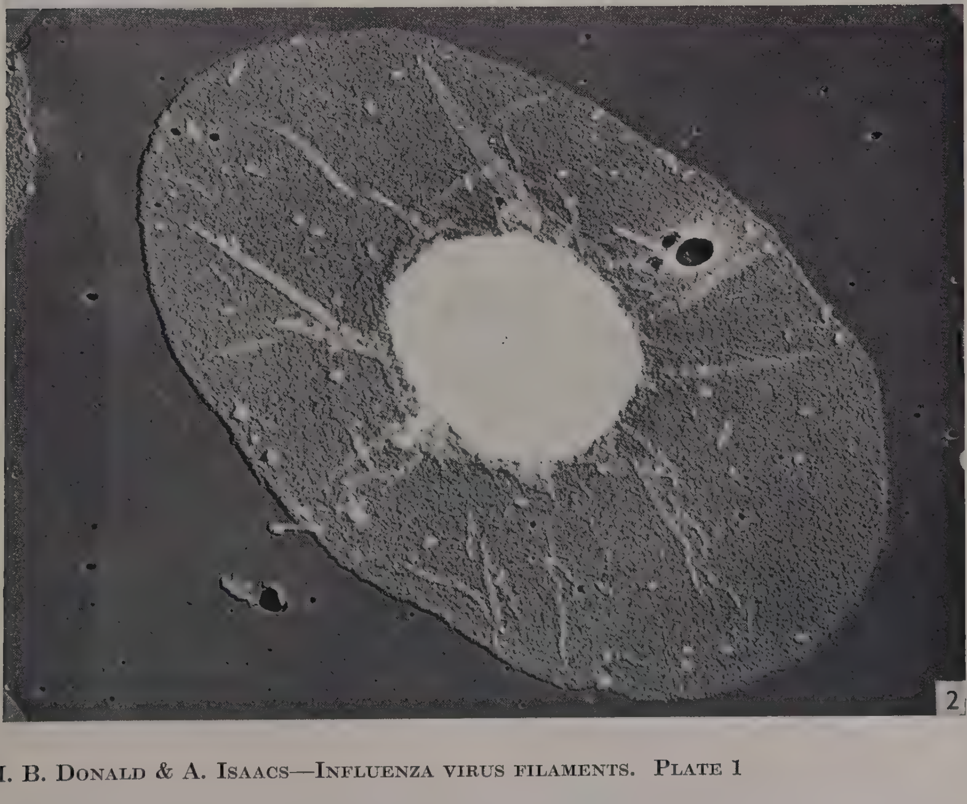
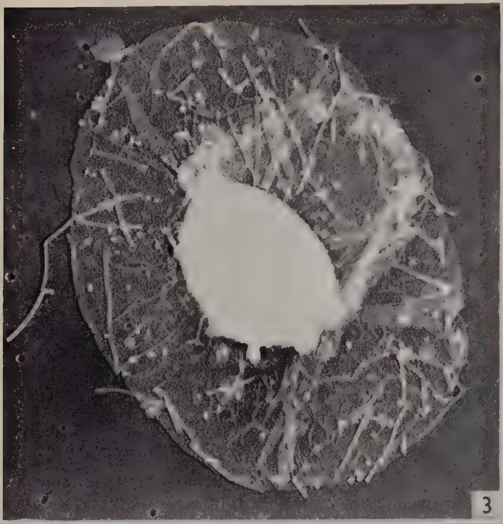


2

H. B. DONALD & A. ISAACS—INFLUENZA VIRUS FILAMENTS. PLATE 1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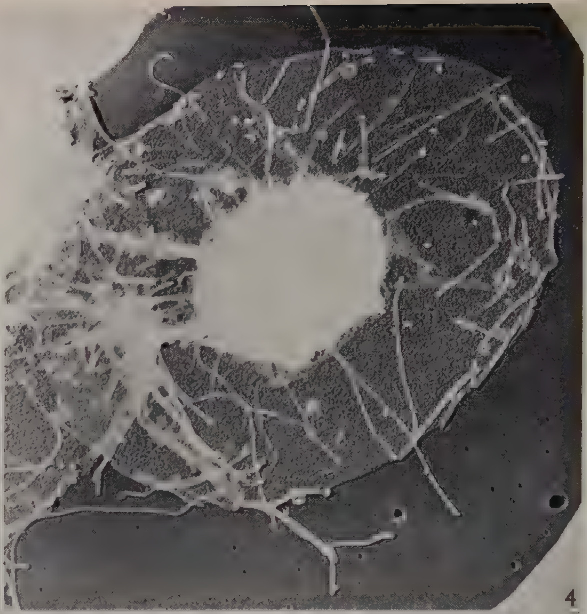
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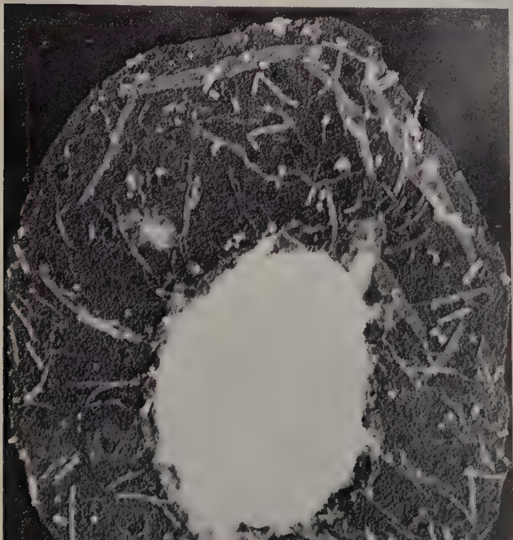
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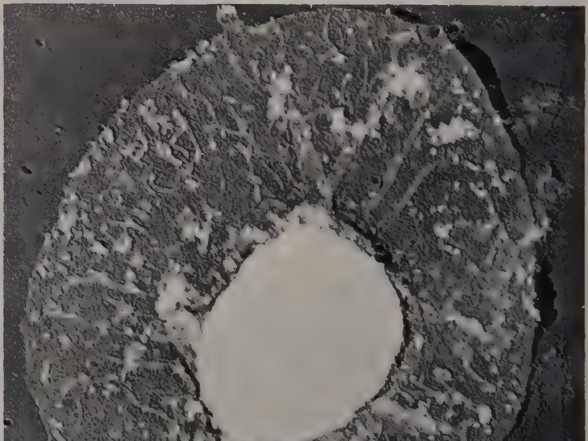
6

H. B. DONALD & A. ISAACS—INFLUENZA VIRUS FILAMENTS. PLATE 2 into spheres is not a simple mechanical process which can be imitated by treatment with ultrasonic vibrations.

We should like to thank Mrs I. M. Honey and Messrs E. Owen and O. Green for their very valuable technical assistance.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Laked fowl erythrocyte with adsorbed A/Persian Gulf/2/52 virus. Shadow cast with gold-manganin. × 12,000.

Fig. 2. A/Persian Gulf/2/52 virus after filtration through gradocol membrane A.P.D. 0·7μ. × 10,000.

Plate 2

Fig. 3. A/Persian Gulf/2/52 virus supernatant after centrifugation at 1500g for 15 min. × 7500.

Fig. 4. A/Persian Gulf/2/52 virus deposit after centrifugation at 1500g for 15 min. × 7500.

Fig. 5. A/Persian Gulf/2/52 virus after treatment with 6 kc. sonic vibrations for 3 min. × 9000.

Fig. 6. A/Persian Gulf/2/52 virus after treatment with 350 kc. ultrasonic vibrations for 3 min. × 9000.

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A Method for Measuring Mutagenic Action

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SUMMARY: A procedure has been evolved which permits an evaluation of mutagenic activity. It appears to be relatively free of experimental bias and analyses the effect of the mutagenic agent more or less independently of selection in the growth medium. It separates the period of phenotypic expression into a distinct unit so that it may be studied separately. Its sensitivity depends on the ability to differentiate cultures containing mutants and its precision depends on the number of flasks employed for subcultures.

In a previous paper Jordan, Mefferd & Wyss (1951) reported that broth cultures, inoculated from suspensions of *Bacillus anthracis* spores irradiated with ultraviolet light, contained mutants in excess of the spontaneous level, providing large enough inocula were used; suitably small inocula of irradiated organisms gave rise to cultures which were normal with respect to their concentration of mutant cells. A given irradiated suspension would produce mutant-enriched subcultures only when the inoculum of the survivors used was large enough to include at least one induced mutant. It seemed likely that a measurement of the minimal volume which produced such mutant-enriched subcultures should be useful as a measure of mutagenic activity. It is required only that the irradiated suspension be diluted to the level where, when a series of broth subcultures is inoculated from it with inocula identical in size, some of the subcultures will contain mutants in excess of the spontaneous level and others will not. When the subcultures grow to dense populations they can be assayed for content of mutants and, assuming a Poisson distribution, the number of potential mutants (cells that will produce at least some progeny of the mutant phenotype) that were in the irradiated suspension can be estimated from the dilution factor and the fraction of the subcultures which contains no more mutants than the level occurring in unirradiated controls. This method permits the phenotypic expression of all potential mutants, it depends for quantitation on a dilution procedure that will serve for estimating diverse types of mutants, it minimizes the influence of selective effects in the subculture on the final result, and it provides a sufficiently large population for detecting rare mutants when mutagenic procedures of a high degree of lethality are employed.

The experimental approach applied in this study was suggested by a similar procedure utilized by Englesberg (1952) to demonstrate the absolute increase in cells of a *Pseudomonas* sp. capable of utilizing itaconate after treatment with formaldehyde and by the procedure of Luria & Delbrück (1943) for estimating the spontaneous mutation rate to phage resistance in *Escherichia coli* strain B.

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METHODS

The organism employed was the Y-53 strain of *Escherichia coli* K-12, a quadruple mutant differing from the parent strain in that it requires leucine, threonine, and thiamine for growth and is unable to ferment lactose (Lederberg, 1947). Suspensions of the organism were grown in brain heart infusion broth (Difco) and incubated at 37° with constant shaking for 24 hr. Suitable samples of the culture were sedimented by centrifugation, washed once and resuspended in sterile water. Ten ml. of the cell suspension containing 5×10^8 cells/ml. were placed in a sterile Petri dish and irradiated for the desired length of time by means of a G.E. Sterilamp (15 w) at a standardized distance. The dish was agitated gently during the period of exposure.

Serial dilutions of the irradiated suspensions were prepared and from each of several dilutions, determined by a preliminary experiment to be near the end point, ten flasks containing 10 ml. of brain heart infusion broth were inoculated and incubated for 24 hr. at 37°. The frequency of mutants in each culture was determined by plating on suitable selective media to determine which flasks were enriched in mutants. From the first term of the Poisson distribution ($P_0 = e^{-M}$ or $M = -\ln P_0$, where P_0 = fraction of cultures having no mutants above the spontaneous, M = average number of induced mutants inoculated/flask) an estimation of the number of mutants induced by the ultraviolet treatment could be made.

Cultures were analysed for the frequency of streptomycin-resistant mutants by plating samples in quintuplicate on streptomycin assay agar containing 5 μg. streptomycin/ml.; the reliability of the procedure was confirmed by assaying known mixtures of resistant and sensitive cells. Mutants capable of utilizing lactose were estimated by plating on a chemically defined medium containing lactose as the major carbon source. The components of the medium were the same as those used by Ryan (1952), except that leucine 40 μg./ml., threonine 40 μg./ml. and thiamine 1 μg./ml. were added. Background growth encountered by Ryan with the ML strain of *Escherichia coli* was not troublesome in the case of *E. coli* Y-53. Recovery experiments made with known mixtures indicated that satisfactory results were obtained when the cultures were examined after 48 hr. Threonine-independent cells were assayed by plating the suspensions on the medium of Davis & Mingioli (1950) containing leucine 40 μg./ml. and thiamine 1 μg./ml. The plates for this mutant assay were incubated at 41° since this appeared to eliminate background growth. Again analysis of known mixtures indicated that satisfactory recoveries could be made.

RESULTS

In the experiment reported in Table 1 about 5·5% of the cells which were subjected to irradiation formed colonies on brain heart infusion agar. The survivors were inoculated into replicate flasks of broth at the rate of $2{\cdot}2 \times 10^6$ viable cells/flask since preliminary experiments indicated that this number would sometimes contain induced mutants. After 24 hr. the flasks, which then contained about 5×10^9 cells/ml., were assayed for mutants. At the levels plated (10^8 cells/plate) no streptomycin-resistant cultures appeared in the unirradiated population, and as can be seen from Table 1 three of the flasks inoculated with the indicated dilution of the irradiated suspension contained no mutants but seven flasks contained mutants in excess of the spontaneous level. From the $P_0 = 0{\cdot}3$ an incidence of induced mutants of 1·2 per $2{\cdot}2 \times 10^6$ irradiated survivors is indicated. For the lactose mutants it appears that values larger than 70 (limits of expectation from Stevens tables: Fisher & Yates, 1949) indicate the presence of induced mutants, and half of the flasks ($P_0 = 0{\cdot}5$) contain such mutants. For threonine independence six of the flasks contained induced mutants. In this method the actual number of the mutants on the plate has no significance except as an indication that it exceeds the spontaneous level, and therefore it need not be determined with precision. The results indicate the independent origin of the induced mutants since flasks containing increased numbers of one type of mutant may contain only the spontaneous level of the others.

The capacities of a known mixture of streptomycin-resistant and non-resistant cells, of an ultraviolet-irradiated suspension and of an untreated suspension to produce cultures containing streptomycin-resistant mutants in excess of the spontaneous level were compared. A marked similarity between the behaviour of the artificial mixture and the irradiated suspension was noted (Table 2). In both cases, cultures prepared from large inocula contained mutants above the normal frequency; however, in both, cultures prepared from smaller inocula did not. In both instances dilutions were found which when inoculated into a series of flasks would give rise to cultures only a fraction of which were enriched with mutants. By contrast cultures prepared from the untreated suspension were found to contain a uniformly low number of streptomycin-resistant mutants.

Because the method being studied depends on the recognition of cultures with mutants in numbers above the spontaneous level, an effort was made to estimate the upper limit of the frequency of streptomycin-resistant cells in cultures prepared from untreated inocula. The values found in the last three lines of Table 2 are usual, and it can be observed that they are not affected by the size of the inoculum. Of 128 such cultures started from various-sized inocula, only five have given values above 1×10^{-9}. This value has been chosen arbitrarily as a value above which the culture is considered to have streptomycin-resistant cells above the spontaneous frequency. The values for the spontaneous level were obtained by plating between 5 and 8×10^9 cells, the final concentration of cells/ml. after 24 hr. growth in brain heart infusion broth. Consequently, between 0 and 8 colonies per plate are usually found, and the zeros reported in Table 1 where 10^8 cells were plated may be regarded as legitimate indication that no induced mutants were present in those flasks. When 1 ml. volumes of the untreated suspension or of the irradiated survivors were plated directly on assay agar at the concentration of streptomycin used, no colonies appeared because in the first instance the incidence of spontaneous mutants was too low and in the latter case the induced mutants required several divisions before the resistance was expressed phenotypically (Demerec,

Table 1. *A comparison of the activity of ultraviolet light for inducing diverse mutations in* Escherichia coli *Y*-53

Mutant character	Value at or below which culture is considered negative	Final incidence of mutants per 10^8 cells after 24 hr. growth										Fraction of cultures without excess mutants (P_0)	Induced mutants/ $2 \cdot 2 \times 10^6$ cells* (M)
Streptomycin resistance	0	100	0	70	100	30	70	40	100	0	0	0·3	1·2
Lactose utilization	70	50	20	50	1680	170	160	560	30	180	70	0·5	0·7
Threonine independence	10	50	5	100	130	20	140	5	2	20	7	0·4	0·9

* This number of survivors of irradiated cells was inoculated per flask.

The values for cultures prepared from ten replicates of untreated suspensions were: streptomycin resistance 0; lactose utilization, range 20–60, mean 42, S.D. 15; threonine independence, range 1–6, mean 3·3, S.D. 1·8.

Table 2. *Reconstruction experiment testing streptomycin resistance*

Sample	Inoculum (ml./flask)	Viable cells inoculated (no./flask)	Incidence of streptomycin-resistant cells per 10^9 bacteria in each flask after growth										P_0	M	Induced mutants (no./ml.)
Artificial mixture	0·1	$1 \cdot 9 \times 10^7$	345	100	243	253	373	109	74	320	260	320	0	—	—
	0·01	$1 \cdot 9 \times 10^6$	0·28	0·65	0·65	0·19	0	330	0·93	0·28	284	440	0·7	0·36	36
	0·001	$1 \cdot 9 \times 10^5$	0·93	0·19	0	0	0·93	1440	0·19	0·19	0	0·19	0·9	0·11	110
Irradiated suspension	0·1	$2 \cdot 5 \times 10^7$	55	330	145	159	415	170	135	66	69	215	0	—	—
	0·01	$2 \cdot 5 \times 10^6$	19·5	4·6	0	356	168	166	176	276	2	0	0·2	1·6	160
	0·001	$2 \cdot 5 \times 10^5$	0·15	0·23	0·15	0·22	0	5·6	0·38	0·1	0·22	0·78	0·9	0·11	110
Untreated suspension	0·1	$2 \cdot 9 \times 10^7$	0·26	0·1	0·78	0·26	0·1	0	0·4	0·2	0	0·1	1·0	—	—
	0·01	$2 \cdot 9 \times 10^6$	0	0·66	0·46	0·2	0·1	0	0·1	0·13	0·1	0·33	1·0	—	—
	0·001	$2 \cdot 9 \times 10^5$	0	0·2	0·46	0·26	0·1	0·2	0·52	0·13	0·1	0·85	1·0	—	—

1951). However, when the 1 ml. of irradiated organism was divided into 0·1 ml. portions and inoculated into ten flasks, after a growth period all of the flasks would contain mutants at an increased level, and when only 0·01 ml. was inoculated eight out of ten subcultures were enriched in mutants. Since no mutants arose when the unirradiated culture was similarly diluted it is clearly evident without recourse to statistics that there was an absolute increase in the number of potential mutants in each millilitre of culture following irradiation.

The precision of the procedure can be increased to any desired degree by employing larger numbers of flasks. The number of streptomycin-resistant mutants in the prepared artificial mixture, estimated by plating directly on streptomycin assay agar, was 62/ml. The values determined experimentally from two dilutions were 36 and 110/ml., respectively. By using the data of several dilutions and 'most probable numbers' tables as used for coliform indices, a maximum likelihood value can be obtained. The sensitivity of the procedure depends on how many bacteria must be plated to detect the spontaneous incidence of streptomycin-resistant bacteria. From the results presented it appears that at least 5×10^9 cells must be plated for even reasonable accuracy. Obviously, if one-tenth as many cells are plated a loss of sensitivity in the detection of positive cultures will occur since cultures with only a slight increase in the frequency of resistant cells may appear negative. However, the mechanics of the method operate against this loss of sensitivity. When 5×10^8 cells/ml. are treated with ultraviolet radiation leaving $2{\cdot}5 \times 10^8$ cells as survivors, and 0·1 ml. of a tenfold dilution is inoculated into each of a series of flasks each flask receives $2{\cdot}5 \times 10^6$ cells and may receive as a minimum 1 potential mutant. This indicates an initial incidence of 400 mutants/10^9 cells. If all descendants of the potential mutant are mutants and if no selection occurs during growth, the final minimal incidence should equal the initial and the plating of as few as 10^7 cells should yield four colonies. Experimentally the observed minimum frequencies are considerably lower than those expected; however, most of those observed in cultures containing mutants above the spontaneous levels have been increased many-fold over the negative cultures. Consequently, a large proportion of enriched cultures can be detected by plating as few as 5×10^7 cells.

This property of the procedure has been utilized in devising a less laborious method for detecting enriched cultures. By placing one drop (*c.* 0·05 ml.) of the culture on the surface of streptomycin-containing agar, spreading over an area of 1 sq.in., and allowing it to dry, approximately $2{\cdot}5 \times 10^8$ cells may be assayed. After incubation, streptomycin-resistant cells are readily distinguished by the development of colonies in the corresponding area on the streptomycin agar. The method was patterned after that employed by Pierce, Dubos & Schaefer (1953) for the detection of tubercle bacilli in the various organs of mice. Comparison of the results indicated that in six experiments representing an analysis of 275 cultures, by plating 1·0 ml. of culture fluid, when only one drop of each culture was used for the analysis 132 had resistant cells in excess of spontaneous level; only six false negatives and one false positive were observed.

Table 3. *A comparison of the activity of two degrees of irradiation for inducing streptomycin resistant mutants*

Period of irradiation (sec.)	Inoculation/flask		Fraction of flasks without induced mutants	Mutants in irradiated suspension (no./ml.)	Induced mutants among survivors (no./10^9)
	Volume (ml.)	Viable cells			
20	0·1	8×10^6	0/10	—	—
20	0·01	8×10^5	7/10	36	450
20	0·005	4×10^5	8/10	45	560
20	0·001	8×10^4	10/10	—	—
40	0·1	$9{\cdot}8 \times 10^4$	5/10	7	7050
40	0·01	$9{\cdot}8 \times 10^3$	10/10	—	—
40	0·005	$4{\cdot}6 \times 10^3$	10/10	—	—
40	0·001	$9{\cdot}8 \times 10^2$	10/10	—	—

Initial number of organisms irradiated was $2{\cdot}7 \times 10^8$/ml. The percentage survival after 20 sec. irradiation was 29·6 % and after 40 sec. irradiation was 0·363 %.

Using this technique of analysis, which eliminates the tedious counting of colonies since visual inspection readily reveals those spots which have colonies in excess of the spontaneous rate, the mutagenic activity of 20 and 40 sec. exposure to ultraviolet light were compared (Table 3). The results were in accord with those expected, in that the absolute number of mutants was higher after the shorter irradiation but fell as the number of survivors decreased. The ratio of mutants to survivors increased with the longer period of irradiation owing to the increments of mutation events.

DISCUSSION

The observation of Jordan *et al.* (1951) that the ability of irradiated anthrax spores to give rise to cultures enriched in mutants was a function of the inoculum size has been extended to another organism and other mutations. It is implicit in the method reported here that there exists in the irradiated suspension, at the time of inoculating the subculture flasks, a variable factor induced by ultraviolet light which leads to the development of excess mutants in only a fraction of a series of flasks equally inoculated from a suitable dilution. This is not affected by the procedure used in assaying for the mutants since the same method shows other flasks to have mutants only at the spontaneous level. The induced mutants exist independently of the testing procedure. The experiments demonstrate that the existence of the streptomycin-resistant mutant is not conditioned by the presence of streptomycin, just as that of the threonine-deficient mutant is not conditioned by the absence of threonine or that of the lactose-positive mutant by the presence of lactose. In further support of this view is the observation that mutation in each of these three loci is independent of the others. The results are consistent with the view that the ultraviolet-irradiated suspension consists of a mixture of diverse potential mutants and non-mutant cells.

The quantitative aspects of the procedure do not depend on the absolute number of mutants found in the mutant assay; factors of selection and competition presumably act on the spontaneous mutants in a manner similar to their action on induced mutants, and it is necessary only that the assay procedure be sufficiently sensitive to distinguish the populations that have developed from inocula enriched in mutants. It would appear that the method should be especially useful in comparing the effect of a given mutagenic procedure for several loci in the same population.

The technique presented here is basically similar to that used by Demerec (1951) for the estimation of mutation to streptomycin resistance in *Escherichia coli*, strain B; in that procedure, irradiated suspensions are spread directly on the surface of nutrient agar and allowed to grow for a short time after which streptomycin is applied in a second layer of agar, the streptomycin-resistant clones grow to full colonies and can be counted. Demerec & Cahn (1953) utilized a slightly modified procedure for the estimation of induced reversions in biochemical mutants. The suspensions treated with the mutagenic agent are plated immediately on minimal media containing a suboptimal enrichment which permits enough growth for phenotypic expression to occur. The method employed in analyzing the mutation from streptomycin dependence to independence (Bertani, 1951) is similar except that background growth in the absence of streptomycin is relied upon to allow phenotypic expression. The amount of background growth in this case depends on the concentration of streptomycin in which the cells were grown before treatment with the mutagen. In each of the last two methods the period of phentoypic expression occurs in a highly selective environment. The need for adequate procedures for estimating the quantitative and qualitative effects of mutagenic agents cannot be overestimated. It is felt that the comparison of results obtained by diverse procedures will do much to clarify the properties of bacterial cells which have been subjected to mutagenic environments.

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The Metabolism of Naphthalene by Soil Bacteria

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SUMMARY: The early stages in the oxidation of naphthalene by five micro-organisms were investigated. In all the organisms, D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, salicylic acid and catechol appeared to be intermediate oxidation products. Washed cells grown on naphthalene oxidized α- and β-naphthol, but whether these compounds are true intermediates remains undecided.

When naphthalene is administered to animals, D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene (naphthalene diol), α-naphthol and their conjugates can be isolated from the urine. The diol and its glucuronide are readily dehydrated in dilute acid solution to naphthol and naphthol glucuronide respectively. Corner, Billett & Young (1954) found that most of the glucuronide in the urine of naphthalene-treated rabbits was diol glucuronide and suggested that the naphthol glucuronide reported by previous workers was an artefact. If this suggestion is well-founded, the scheme of naphthalene metabolism presented by Boyland & Wiltshire (1953*b*) can be simplified by omission of the pathway naphthalene → α-naphthol, and naphthol can be excluded as an intermediate in naphthalene oxidation by animals. Such a conclusion would be consonant with the conversion of naphthalene to diol, but not to naphthol by rat liver slices (Boyland & Wiltshire, 1953*a*). Walker & Wiltshire (1953) showed that a certain soil bacterium oxidized naphthalene to naphthalene diol but did not produce or oxidize naphthols. Several different pseudomonads which had been grown with naphthalene as sole carbon source gave an immediate oxygen uptake with α- or β-naphthol as substrate (Stanier & Sistrom, personal communication). Treccani (1953) studied another naphthalene-utilizing organism, probably a *Nocardia* species, which produced salicylic acid from naphthalene.

We have therefore compared the pathways of naphthalene catabolism in five different organisms using growth in defined media, the simultaneous adaptation technique (Stanier, 1947) and chemical methods for the identification of intermediate oxidation products. The organisms used were: two Pseudomonas strains, 53/1 and 53/2, kindly supplied by Dr Sistrom; the organism described by Walker & Wiltshire (1953) and here referred to as organism I; a Pseudomonas strain isolated from Rothamsted soil (organism II); a Nocardia strain R, isolated and described by Treccani (1953).

Organism II is a motile Gram-negative rod which produces a greenish fluorescence in nutrient agar. It forms smooth circular greyish colonies on peptone agar and an even turbidity in peptone water. Nitrite is produced from nitrate, and acid but no gas from sucrose, glucose or maltose. It does not liquefy gelatine and it can attack phenol and naphthalene. This organism grows well at 25° and thus closely resembles *Pseudomonas desmolyticum* described by Gray & Thornton (1928).

METHODS

Media. The following basal inorganic media were used with the relevant organic substrate:

Medium A. Tausson's (1927) medium with or without the addition of agar, and compounded from solution *a* ($Ca(NO_3)_2$, 1 g.; KNO_3, 0·25 g.; $MgSO_4.7H_2O$, 0·25 g.; $FeCl_3$, 2·5 mg.; distilled water, 200 ml.) and solution *b* (KH_2PO_4, 0·08 g.; K_2HPO_4, 0·2 g.; distilled water, 200 ml.) sterilized separately and mixed aseptically in the proportion 4 parts *a* to 1 part *b*.

Medium B. KH_2PO_4, 1 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4$, 1 g.; $CaCO_3$, 0·1 g. in 1000 ml. tap water, pH 6·8–7·0.

Cultural conditions were as described by Walker & Wiltshire (1953) except that sometimes media containing also 1% of crystalline sodium succinate were used.

Manometric method. Organisms were washed in 0·02 M-phosphate buffer (pH 6·98) and resuspended in the same buffer. The concentration of the suspensions was standardized turbidimetrically by means of a photoelectric absorptiometer. The oxygen uptake by 1 ml. cell suspension (containing about 0·3 mg. total-N) was measured in Warburg manometers at 30°. The main cup of the Warburg flask contained the cells and 0·02 M-phosphate buffer (pH 6·98) to give a total volume of 3 ml., the substrates being placed in the side bulb. The centre cup contained 0·2 ml. 20% (w/v) aqueous potassium hydroxide.

Detection of intermediates. Reactions for the detection of naphthalene diol, naphthols and salicylic acid were as described previously (Walker & Wiltshire, 1953). Catechol was detected as described by Evans (1947).

RESULTS

Growth experiments

To test whether they could serve as carbon sources for growth, the following substances, in sterile solution to give the final concentrations shown, were added to 25 ml. volumes of medium B in 100 ml. Erlenmeyer flasks: sodium succinate (0·5%), sodium salicylate (0·25%), catechol (0·02%), α-naphthol (0·001%), β-naphthol (0·001%). Growth took place on naphthalene, salicylate or succinate but not on catechol or the naphthols. Although there was no growth on catechol, this compound was detected in all cultures growing on salicylate. Viable cells were still present in the naphthol-containing cultures at the end of the experiment (8 days). In medium A neither strain R nor organism II grew on the naphthols at 0·003% or 0·006%.

A further experiment was carried out using strain 53/1, naphthalene-grown cells of which had been found to oxidize both naphthols rapidly. Naphthalene-grown cells of strain 53/1 were inoculated into 50 ml. lots of medium A in 500 ml. Erlenmeyer flasks to which α-naphthol, β-naphthol or salicylic acid was added to give a final concentration of 0·02%, 0·01% or 0·0067%; control flasks contained no added carbon compound. Only salicylic acid permitted growth though the cells remained viable up to at least 10 days in the naphthol cultures as was verified by making transfers into nutrient broth.

Detection of intermediate products. Various products were detected in liquid cultures containing naphthalene, grown in either Roux bottles without agitation or in flasks with stirring. We have already reported the isolation of naphthalene diol and salicylic acid from cultures of organism I in medium A (Walker & Wiltshire, 1953); catechol was also detected in the present work. Naphthalene diol, salicylic acid and catechol were all found in cultures of organism II and of strains 53/1 and 53/2 in media A and B. Strain R accumulated the same compounds in medium A, but in medium B no diol was found. In no case was a naphthol detected at any stage of growth.

Comparison of oxygen-uptake of organisms grown on succinate and naphthalene

All five strains were grown on plates of medium A solidified with agar and containing naphthalene, succinate or a mixture of naphthalene and succinate, in order to compare naphthalene-adapted and non-adapted cells. Organisms grown on succinate + naphthalene behaved like those grown on naphthalene. Strains 53/1 and 53/2 were indistinguishable in their behaviour. When grown on succinate none of the strains immediately oxidized naphthalene or any of the postulated intermediates, although in the case of organism II there was only a short lag before the cells began to oxidize naphthalene. When grown on naphthalene or a mixture of naphthalene and succinate, all strains oxidized immediately naphthalene, naphthalene diol, salicylic acid and catechol; in addition strains 53/1 and 53/2 oxidized α- and β-naphthol. Results typical for organisms I and II and strain R are shown in Fig. 1 A and for strains 53/1 and 53/2 in Fig. 1 B. The oxygen uptake by washed cells alone has been deducted, and the uptake with β-naphthol is omitted because it was much the same as for α-naphthol.

Oxidation of naphthols

When grown on naphthalene, organism II was very sensitive to small changes in α-naphthol concentration, with a maximum rate of oxidation at $1{\cdot}33 \times 10^{-3}$ M. The optimal concentration for organism I and strain R was lower (Fig. 2 A, B). Strain 53/1 oxidized naphthols more rapidly than any other strain, and it was not inhibited by any concentration of naphthol used. However, cells of this strain, when grown on succinate alone, also oxidized naphthols at a suitably low concentration, the respiration being slightly inhibited by higher concentrations (Fig. 3).

Inhibition of oxygen uptake by naphthols

Naphthols at $1{\cdot}66 \times 10^{-3}$ M inhibited the oxidation of naphthalene by adapted cells of strain 53/1, organism I or organism II (Fig. 4). β-Naphthol was less inhibitory than α-naphthol especially in the case of strain 53/1. Salicylic acid at the same concentration did not inhibit. The oxygen uptake by salicylate-grown cells with salicylate as substrate was also inhibited, especially by α-naphthol, but not at all by naphthalene.

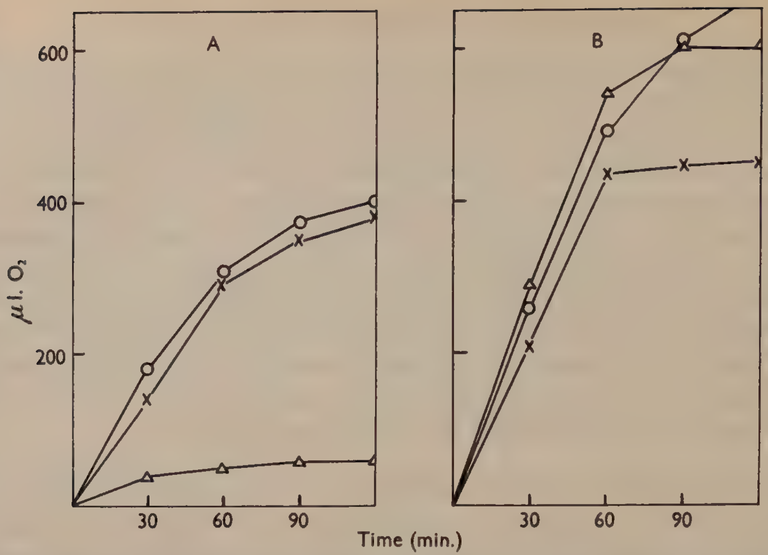

Fig. 1. Comparison of different naphthalene-oxidizing bacteria. Rate of oxygen uptake by washed cells in 0·02 M-phosphate buffer pH 7·0 in presence of: 5 μmole naphthalene (○——○), salicylic acid (×——×), α-naphthol (△——△); A, organisms I, II and R; B, strains 53/1 and 53/2.

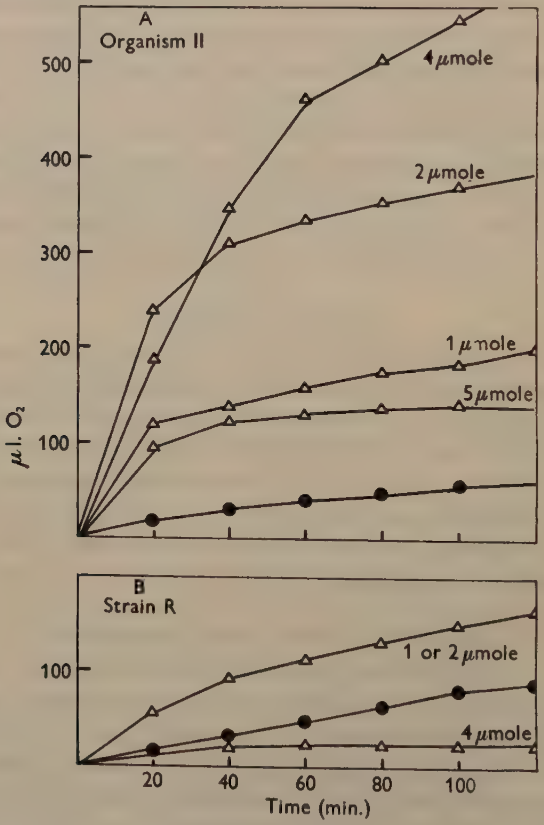

Fig. 2. Effect of naphthol concentration on the rate of oxygen uptake by naphthalene-adapted cells. Oxygen uptake by washed cells (●——●) and in presence of several concentrations of α-naphthol (△——△). A, organism II; B, strain R.

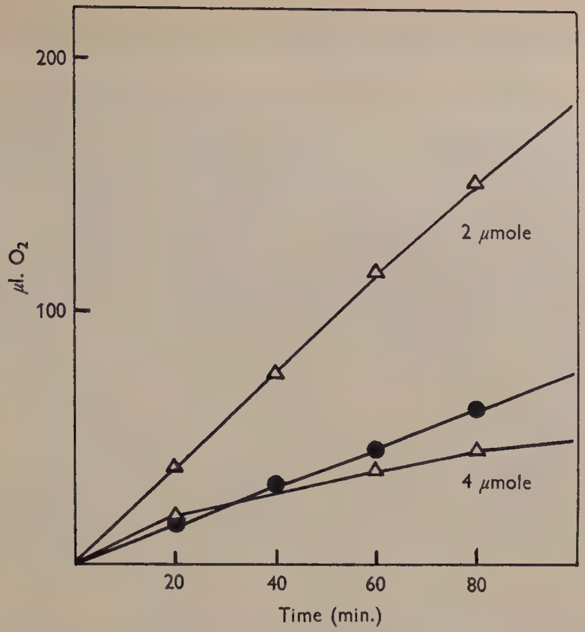

Fig. 3. Effect of naphthol concentration on the rate of oxygen uptake by succinate-grown cells of strain 53/1. Oxygen uptake by washed cells (●——●) and in presence of two concentrations of α-naphthol (△——△).

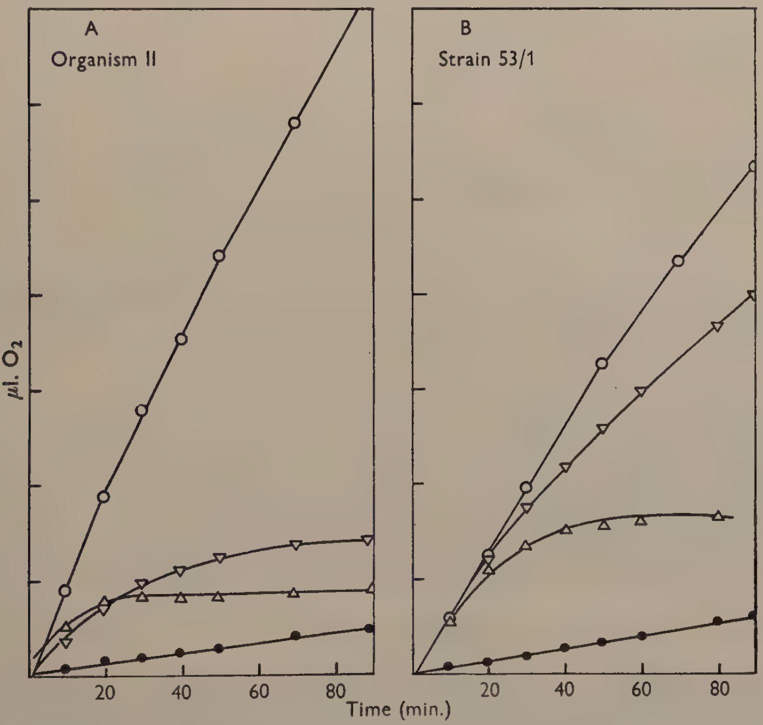

Fig. 4. Inhibition by naphthols of the oxidation of naphthalene. Oxygen uptake by naphthalene-grown cells of (A) organism II and (B) strain 53/1 (●——●) and in presence of 8 μmole naphthalene (○——○), 8 μmole naphthalene + 5 μmole α-naphthol (△——△), and 8 μmole naphthalene + 5 μmole β-naphthol (▽——▽).

Inhibition of oxygen uptake by β-naphthoquinone

The ability of organism II to oxidize low but not high concentrations of naphthols suggested that both naphthols might be converted to some product having a toxic effect on the cells. Non-enzymic oxidation might produce 1:2-naphthoquinone or the related 1:2-dihydroxynaphthalene. Neither of these compounds at 10^{-4} to 10^{-3} M was oxidized at all rapidly by organism I (Walker & Wiltshire, 1953). There was no oxygen uptake by organism II with naphthoquinone in this range of concentration (Fig. 5) but instead a marked inhibition of the endogenous respiration. Moreover, 1:2-naphthoquinone decreased the oxygen uptake with $1{\cdot}33 \times 10^{-3}$ M-α-naphthol. Since it is known that primary aromatic amines can react with quinones to form insoluble indirect quinonoid addition compounds, an attempt was made to abolish the naphthoquinone inhibition by aniline; this attempt was unsuccessful however. Also, aniline failed to abolish the inhibition by α-naphthol of the oxidation of naphthalene.

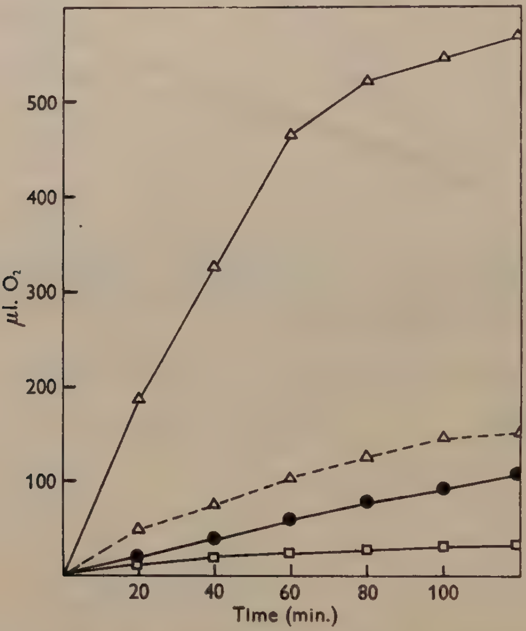

Fig. 5. Inhibition by β-naphthoquinone of the oxidation of α-naphthol by naphthalene-adapted cells of organism II. Oxygen uptake by washed cells (●——●), and in presence of 4 μmole α-naphthol (△——△), 1 μmole β-naphthoquinone (□——□) and a mixture of 4 μmole α-naphthol and 1 μmole β-naphthoquinone (△- - -△).

DISCUSSION

To decide whether a given compound is an intermediate in the metabolism of a substrate by bacteria, it is desirable to use as many criteria as possible, for example: detection or isolation of the intermediate from growing cultures (the most convincing evidence); growth of the given organism on the supposed intermediate; the intermediate compound should be oxidized as rapidly as the initial substrate by adapted cells.

In the case of naphthalene metabolism, naphthalene diol, salicylic acid and catechol were isolated or detected in the cultures of all the five organisms we used, and all these substances were oxidized immediately by naphthalene-grown cells, so that there seems little doubt that they are all true dissimilatory products of naphthalene. All five organisms grow on salicylic acid; the failure to grow on catechol may be attributed to the toxic effect of *o*-benzoquinone.

In the case of α- and β-naphthol, we have only the positive evidence of simultaneous adaptation to support the view that these compounds are intermediates. We have not been able to detect free naphthols in any cultures although the reaction used is sufficiently sensitive to detect 1 p.p.m. of α-naphthol. It is of course conceivable that naphthols do not accumulate even to this extent. Neither naphthol supported the growth of any of the organisms. When the concentration of naphthols was low, there was a moderate rate of oxidation of both naphthols by succinate-grown cells of strain 53/1, but the rate was increased by adaptation to grow on naphthalene. It is unlikely that both α-naphthol and β-naphthol are intermediates since enzymes generally exhibit a high degree of steric specificity; it would be strange to find no discrimination in this case. However, an alternative mechanism may be advanced. Boyland & Sims (1953) found that synthetic naphthalene diol yielded both α-naphthol and β-naphthol on treatment with dilute mineral acid. Since the diol is a rather reactive compound it is possible that some of it, once formed in the culture, might be non-enzymically converted to both naphthols, and the cells would then be incidentally exposed to the two naphthols. Against this hypothesis, it should be repeated that we have never detected naphthols in the cultures. Moreover, Boyland & Sims (1953) stated that the diol produced by animals yielded exclusively α-naphthol when boiled with 2N acid. It is strange that there should be this difference in behaviour between a natural product and the synthetic racemic form. More probably diol always yields both naphthols but in different proportions according to the conditions of reaction.

The main difference between the organisms examined can be attributed to varying susceptibilities to toxic effects of the naphthols; when allowance for this is made, the general behaviour of the organisms is similar. The results of manometric experiments with adapted cells suggest that the enzyme system produced in response to growth in the presence of naphthalene and responsible for oxidation of naphthols is an adaptive one. This remains the only evidence supporting the view that α- or β-naphthol is an intermediate in the bacterial metabolism of naphthalene.

We are indebted to Dr H. G. Thornton, F.R.S., for his interest in this work.

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The Urea Method for Bacterial Viability Counts with the Electron Microscope and its Relation to other Viability Counting Methods

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SUMMARY: Live rod-shaped bacteria incubated on a medium containing 3 % urea grow but do not divide. They thus become much longer than dead bacteria lying among them, and the numbers of each kind can easily be counted in an electron microscope. This urea method gives accurate results and has several advantages over colony-counting methods. The significance of various interesting differences in the counts given by the two methods is discussed. Slope cultures at 18 hr. gave live counts of over 95 % by the urea method.

A common need in quantitative bacteriology is to discover what proportion of bacteria in a given sample is alive. The standard procedure is to make counts of the colonies which form when measured volumes of the sample, suitably diluted, are incubated in nutrient media. On the assumption that each colony arises from one live bacterium, the number of the latter in the sample can then be determined. Such counts made before and after some treatment will enable the resulting fall in viability to be measured. In order to find the actual percentage of live cells in a sample a total count of the bacteria in the measured volume must also be made. In studying the survival of bacteria during drying from tiny droplets or thin films, an accurate measurement of the volume of the samples becomes difficult or impossible. To overcome this problem a new method has been developed for determining the proportion of live bacteria in such tiny samples. This paper gives details of the method and typical results of a test of its accuracy.

The method is based on the property possessed by urea in concentrations of about 3 % (0·5 M) of preventing the division of certain bacteria without inhibiting their growth (Wilson, 1906). Hence after incubation of suitable bacteria for a few hours on nutrient agar containing urea, the live cells have grown into giant forms which can readily be distinguished from the shorter dead organisms (Pl. 1, fig. 5). The numbers of live and dead can then easily be counted in an electron microscope.

EXPERIMENTAL

The action of urea

We have no information about the mechanism by which urea exerts its effect. We have, however, investigated its action on the growth of a paracolon bacillus over a range of concentrations of urea from 1 to 10 % in nutrient agar medium. The bacteria were taken from 18 hr. slope cultures, suspended in broth, and grown on filmed electron microscope specimen grids as described below.

In the presence of 1 % urea, there was extensive growth with the formation of colonies (Pl. 1, fig. 2), but no giant long forms. Between 1 and 2 % urea, varying proportions of long forms and colonies were produced. With a urea concentration of 3 %, up to as many as 99·5 % of the bacteria grew into long forms (Pl. 1, figs. 3, 4) and no colonies were formed. Concentrations of urea above 4 % produced increasing numbers of bacteria showing neither growth nor division.

Various other agencies for producing long forms were also investigated (lithium salts; ultraviolet and X-irradiation), but none of these was found as satisfactory as urea for producing, as nearly as possible, complete inhibition of division while permitting every live organism to grow.

Description of the method

The medium. A volume of 20 ml. of melted nutrient agar (Difco) is poured into a Petri dish containing 0·6 g. urea, gently rocked to ensure thorough mixing, and then left to set. Care must be taken to avoid contamination of the medium, but it is inadvisable to attempt heat sterilization of the urea agar as there is risk of decomposition of urea with the formation of ammonia.

Choice of bacteria. A species suitable for counting by the urea method must conform to the following requirements: (1) it must grow without cell division on the urea medium; (2) long filamentous forms must not occur normally; (3) it must be capable of appreciable growth in a few hours.

A convenient organism which we have used extensively for the 'urea count' is the paracolon bacillus 'D.201 H', used by Fry & Greaves (1951). On an 18 hr. nutrient agar slope this exists as a short rod (1–3 μ. long) often appearing almost spherical in shape (Pl. 1, fig. 1). But when cultivated on the urea medium the short rods rapidly form filaments (Pl. 1, figs. 3, 4) which may grow to 150 μ. in length. The following organisms have also been tested and all were found suitable for urea counts: *Escherichia coli* (ten untyped strains), *Serratia marcescens* (*Chromobacterium prodigiosum*), *Salmonella paratyphi*-C (Pl. 2, fig. 7), *Proteus morganii* (Pl. 2, fig. 8). All of these produced on urea agar long forms which were easily distinguishable from the normal cells in each case. The long forms of *P. morganii* developed despite the appreciable urease activity possessed by this organism (*Bergey's Manual*, 1948; Ishikawa, 1928), probably because the bulk of medium was so great compared with that of the bacteria. Cocci, however, do not grow long.

The grids. Stainless steel electron microscope specimen grids (R.C.A. Photophone Ltd., 36, Woodstock Grove, London W. 12) were used. Those made of copper or silver may prove toxic on the urea medium. After being well washed in distilled water, the grids were covered with a thin nitrocellulose film (Cosslett, 1951). A drop (0·01 ml.) of 1 % (w/v) nitrocellulose in amyl acetate solution spread on a clean water surface about 50 cm.^{2} in area produces a film of suitable thickness. Too thick a film impedes the diffusion of nutrients during the subsequent incubation of the bacteria on the urea agar; too thin a film tears very easily.

Procedure. Each grid is placed film-side downwards on the medium and a small loopful of the bacterial suspension under investigation is placed centrally upon it, so that the drop covers about two-thirds of the surface of the grid. The bacteria are thus separated from the medium by the film of nitrocellulose (Fig. 1). However, a film prepared as described will allow nutrient material to pass through to the cells (Hillier, Knaysi & Baker, 1948; Brieger & Cosslett, 1949). The dish is then incubated at 37° for a sufficient time to allow all the live bacteria to grow long. With an active suspension this is usually about 3 hr. (Pl. 1, fig. 5), but the time required will be longer when some of the cells, though live, have an increased lag phase because of previous treatment such as drying or irradiation. A suitable time must be found for each experiment, but it should not be longer than *c.* 6 hr., as some lysis will then occur, making the count more difficult.

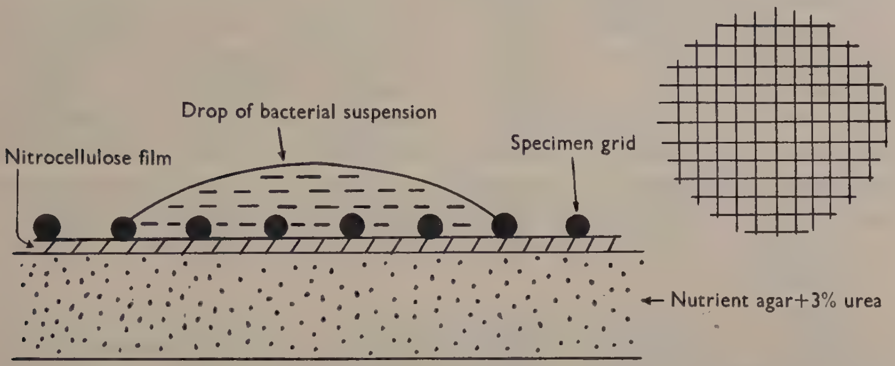

Fig. 1. Diagram (not to scale) showing the incubation of a drop of bacterial suspension on an electron microscope specimen grid placed on the urea agar medium. On the right is a smaller plan view of the grid alone. The actual grids used contain more squares than shown in the diagrams. The grids are 2 mm. in diameter, with bars 70 μ. apart (centre to centre).

After incubation, the bacteria are fixed by inverting the dish over a few drops of formalin or 1 % osmium tetroxide solution for 5 min. The grids are then removed from the medium by running a little distilled water round each from a pipette and allowing them to float off. They are then floated, still film-side downwards, on the surface of distilled water and left overnight. All traces of medium diffuse away leaving very little contaminating material round the bacteria lying on the film. The grids are lifted off the water, lightly blotted at the edge, and allowed to dry completely lying film upwards on filter paper. (The films will tear if they dry in contact with a surface.) The grids are then ready for examination in the electron microscope; there is no need for metal shadowing.

Counting. For accurate counting it is desirable to have available a direct magnification of about 4000 times; below this it is often difficult to distinguish dead bacteria from particles of detritus. We have thus found it essential to use an electron microscope. This also greatly decreases the strain of protracted counting on large numbers of samples. Moreover, empty cell membranes are easily detected in the electron microscope (Pl. 1, figs. 5*b*, 6) so that the lysed cells which commonly result during killing experiments are not overlooked. For the magnifications involved, the simplest models of electron microscope are adequate and handling and alignment are very easy.

A grid of ungrown bacteria is first examined in the electron microscope and the size of the longest normally occurring cell is noted by reference to a simple scale scratched on the fluorescent screen. All those bacteria incubated on the urea medium which have grown longer than this are counted as live. The short ones which have shown no signs of growth are reckoned as dead. When the grid has been incubated for a sufficient time, there is a very clear distinction between the long and the short forms (Pl. 1, fig. 5). This has been confirmed by plotting length distribution curves. When it is necessary to incubate for longer than usual, to allow cells with an increased lag phase to grow, some of the bacteria may be very long and entangled (Pl. 1, fig. 4). In such cases it is easier to count the ends of the long forms and divide by two than to count individual long bacteria. Where ends are counted, one must also count places where a long bacterium meets a grid bar, except where it obviously curls back into the square. Various minor errors may occur (long bacteria surmounting a grid bar so as to lie in two squares; small bacteria hidden beneath the overhanging edge of a grid bar), but the accuracy which can be achieved (see below) indicates that such errors are very small.

The number of cells which must be counted depends on the precision which is desired in the estimate of the percentage of live cells. The standard error of this percentage is inversely proportional to the square root of the number of bacteria counted (provided there is a random distribution of live and dead cells) and is given by the expression

$$\sigma = \pm \sqrt{\left[\frac{L}{n}(100-L)\right]}\,\%,$$

where L is the estimated percentage of live cells, n = the number of cells counted, and σ is the standard error of the percentage L.

Thus with a survival of 50 %, the standard error with a count of 400 cells will be $\pm 2\frac{1}{2}$ %. This therefore will be usually a sufficient number to count on each grid, and if the original drop of bacterial suspension was of a suitable concentration (about $10^7 - 10^8$ bacteria/ml.) the count can be quickly made. We usually count about 400 cells on each of three grids prepared from the same suspension and total the results obtained.

Tests on the validity of the method

The validity of the urea method of counting was tested by using it to determine the live count of bacterial suspensions containing known percentages of heat-killed bacteria.

An 18 hr. agar slope growth of a paracolon bacillus (D. 201 H) was suspended in broth (about 10^7 bacteria/ml.). A few ml. of this suspension were then held at 70° for 5 min. After cooling, the heat-killed suspension was mixed in various proportions with the unheated suspension to give samples containing 100, 75, 50, 25, 12½ and 0% unheated bacteria. (These cannot be taken as exactly the concentrations of live bacteria since the unheated suspension contains some dead cells—see discussion.) Counts were then made by the urea method as described above, three grids being prepared and counted from each suspension. The results are shown in Table 1. The last column gives the counts of long (i.e. 'live') bacteria as percentages of the live count on the unheated cells, and these agree well with the percentages of unheated cells in the suspension.

Table 1. *Results of a test on the accuracy of the urea method*

Counts of live cells in bacterial suspensions prepared by mixing known proportions of heat-killed and unheated organisms; paracolon bacillus D. 201 H.

Unheated cells in sample (%)	Grid no.	Total no. of bacteria counted	Count of long (=live) bacteria (%)	Standard error expected in sample (%)	Count as % of count on 100% unheated cells* (%)
100	1	400	98·5	±0·6	—
	2	450	98·7	±0·5	—
	3	300	98·0	±0·8	—
	Total	1150	98·4	±0·4	100
75	4	890	74·1	±1·5	75·4
	5	883	72·0	±1·5	73·2
	6	923	71·7	±1·5	72·9
	Total	2696	72·7	±0·9	73·9
50	7	(Film torn)			
	8	637	48·5	±2·0	49·3
	9	603	48·5	±2·0	49·3
	Total	1240	48·5	±1·4	49·3
25	10	(Film torn)			
	11	365	24·4	±2·3	24·8
	12	407	23·3	±2·1	23·7
	Total	772	23·8	±1·5	24·2
12½	13	78	16·7	±4·2	17·0
	14	143	13·3	±2·8	13·5
	15	410	12·4	±1·6	12·6
	Total	631	13·1	±1·3	13·3
0		No live bacteria			

* Obtained by increasing the actual live count (column 4) by a factor 100/98·4 in order to take account of the fact that the 100% unheated cells gave a live count of only 98·4%.

DISCUSSION

Advantages and disadvantages of the method. The urea method provides a simple and convenient way of discovering the viability of a bacterial suspension and has a number of advantages over other procedures.

(1) The count is unaffected by clumping of the cells. When a colony count is used to determine the viability it has to be assumed that each colony arises from a single cell, and clumping of the organisms may vitiate the count. That a considerable amount of clumping does exist even in young cultures of *Escherichia coli* after shaking (Jennison, 1937) is a fact that is often overlooked. It is hard to avoid and may be a source of serious error in the usual counting methods. Lea (1946) pointed out that the controversial departures from an

exponential fall in the number of survivors during irradiation may be due to errors in the count as a result of clumping. Migaki & McCulloch (1949), who worked with bacteria treated with surface-active agents, also found the colony-counting method unsatisfactory because of clumping.

(2) Since only a very small unmeasured volume of culture (very approximately 10^{-4} ml./grid) is needed for the count, experiments can be carried out on the survival of bacteria in droplets and films, which have not been possible before. In nature, bacteria are normally carried in these conditions and the results of such experiments should be more relevant than similar ones performed on bulk suspensions.

(3) It is a simple matter to examine the morphology of the bacteria with the electron microscope while the counts are being made. The short (i.e. 'dead') bacteria may differ in appearance from the normal organisms and hence make it possible to associate morphological changes with death.

(4) The urea method is at present the only simple procedure for discovering directly what *percentage* of the bacteria in a culture are alive. The colony count gives the number of viable cells/unit volume of suspension and the *percentage* viability can then be deduced only if a total count is made.

The main limitations of the method have already been noted. It can be applied only to certain bacteria (in particular it is not suitable for cocci), and it requires that the live bacteria shall be capable of showing growth within *c.* 6 hr. The urea method assumes rather different definitions of 'live' and 'dead' bacteria from those adopted in making a colony count, and it may not therefore be suitable for work where the main interest centres on the ability of cells to multiply. This point is further discussed below.

The meaning of 'live' and 'dead' bacteria. In making a colony count, a cell is counted as 'live' only if it can multiply to form a visible colony of bacteria, whereas in the urea method a bacterium, to be reckoned as 'live', is required only to be capable of some growth on the medium. It has been found that there are marked differences between counts made by the two methods on suspensions of bacteria irradiated with X-rays or with ultraviolet light. These differences result from the presence after irradiation of bacteria capable of growth but not of repeated division, which will thus be counted as 'dead' by the colony count and 'live' by the urea method. Such bacteria are also known to exist in cultures treated with antibiotics. It is perhaps best to use the word 'viable' to describe cells which can multiply to form a colony, and the word 'live' more broadly for those which can grow (or show other signs of life such as respiration) even if unable to go on dividing. In many cases, however (e.g. counts on natural untreated populations of bacteria) there is no reason to expect significant numbers of bacteria to be capable of growth but not of division. In these cases colony-counting methods and the urea method are, in principle at least, of equal merit, but the latter is probably more accurate because of the great difficulty of avoiding clumping in the colony-counting method. But, if there is a significant difference between the two counts (clumping having been avoided), and if only those bacteria which can multiply are of interest, then the colony count is the more relevant—though it must be remembered that some of the 'dead' cells may not irreversibly have lost their ability to divide (Heinmets, Taylor & Lehman, 1954). In general it is clearly desirable to make, in appropriate cases, both kinds of count and to compare the results. For instance, in studying the effects of external factors on bacterial cells it is important to distinguish between effects on the systems responsible for producing new protoplasm and effects on the systems involved only in cell division. Comparisons between urea counts (depending on growth alone) and colony counts (depending on both growth and division) enable precisely such a distinction to be made. Moreover, the differences often observed between the results of the two counts confirm the concept, suggested by restoration experiments, that many cells found to be non-viable by colony counts are in other senses still live and can be restored by suitable treatment to full viability (Heinmets *et al.* 1954). The need which these authors indicated for a method of measuring degrees of 'complete' sterility may in fact be met by the urea method.

The percentage viabilities of untreated bacterial cultures. The results given in column 4 of Table 1, which show an average of 98·4 % live cells in suspensions containing 100 % unheated cells, have direct bearing on the question of what proportion of the cells in an actively growing culture is alive. Differing views have been put forward on this point. By comparing the viable count given by the colony counting method with a direct microscopic count of the total number of bacteria, several workers (Wilson, 1922, 1926; Régnier, David & Kaplan, 1932; Jordan & Jacobs, 1944) concluded that usually only about 70–90 % of the organisms are viable even during the logarithmic phase of growth. This is further discussed in *Topley and Wilson* (1946). Kelly & Rahn (1932) found by microscopic observation that, under favourable conditions, all the cells in a culture appeared to divide. Ziegler & Halvorson (1935) stated that when great care was taken to break up clumps of bacteria the colony count and direct count gave the same result. Jennison (1937) concluded that the reported differences between the two counts could be wholly explained by clumping of cells. After measuring and correcting for this he reported that cultures of *Escherichia coli* were practically 100 % viable.

By using the urea method we have consistently found that 95–100 % of the bacteria taken from 18 hr. agar slope cultures of the paracolon bacillus used and of *Escherichia coli* were live. This observation would not be incompatible with the former viewpoint if there were numbers of cells on the slope cultures capable of growth and perhaps a few divisions but, as a result of some disturbance of their division mechanism, incapable of continuing to grow and divide to form a visible colony. However, there is no reason to expect this, and we think it much more probable that the differences between viable counts and total counts have been due to clumping of cells. Thus it may be that, in some cases at least, the viability of an agar slope culture is really much nearer 100 % than colony counts, in conjunction with total counts, have suggested. This would confirm the point made by Hinshelwood (1946) that the close approximation found in practice to a logarithmic growth law suggests that mortality under favourable conditions is low.

We are much indebted to Dr R. M. Fry for supplying us with a culture of the paracolon bacillus (D. 201 H) used by Fry & Greaves (1951); also to the Agricultural Research Council for support during this work.

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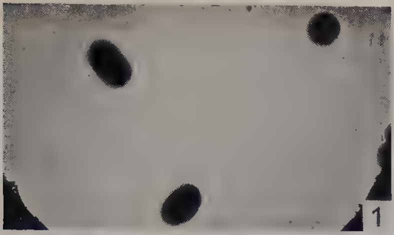
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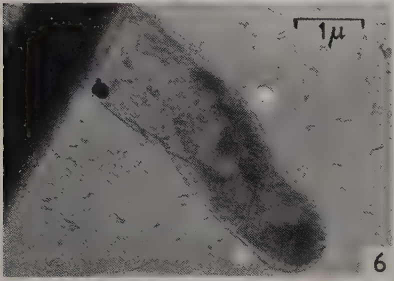

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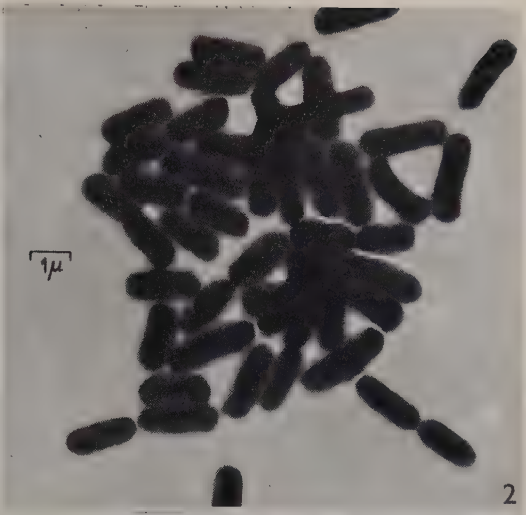

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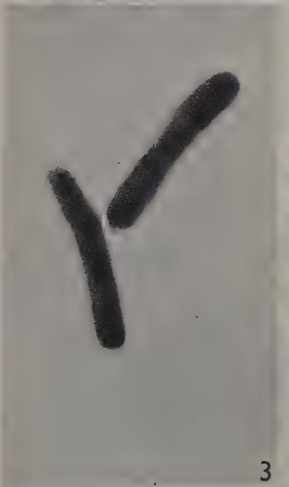
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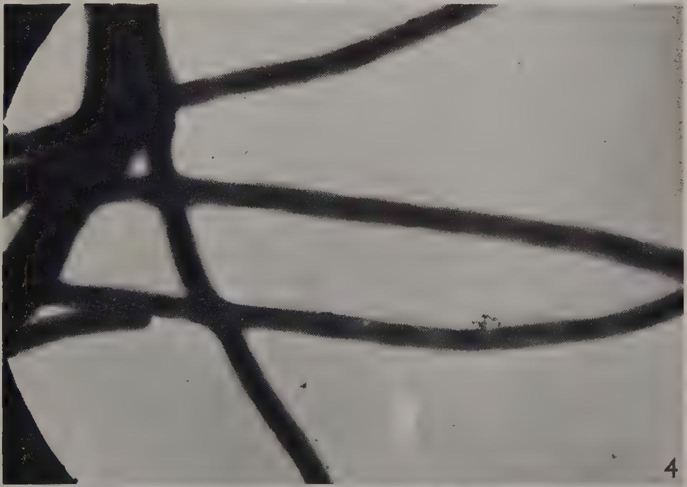
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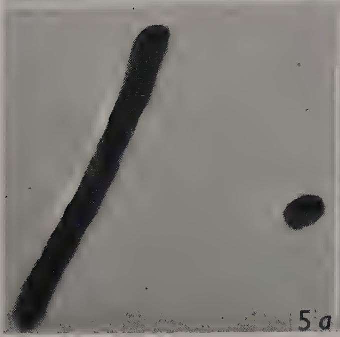
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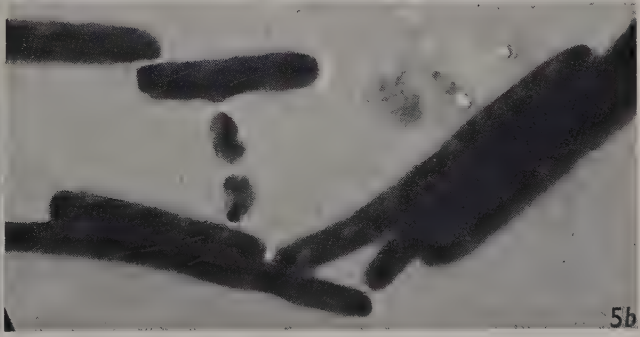
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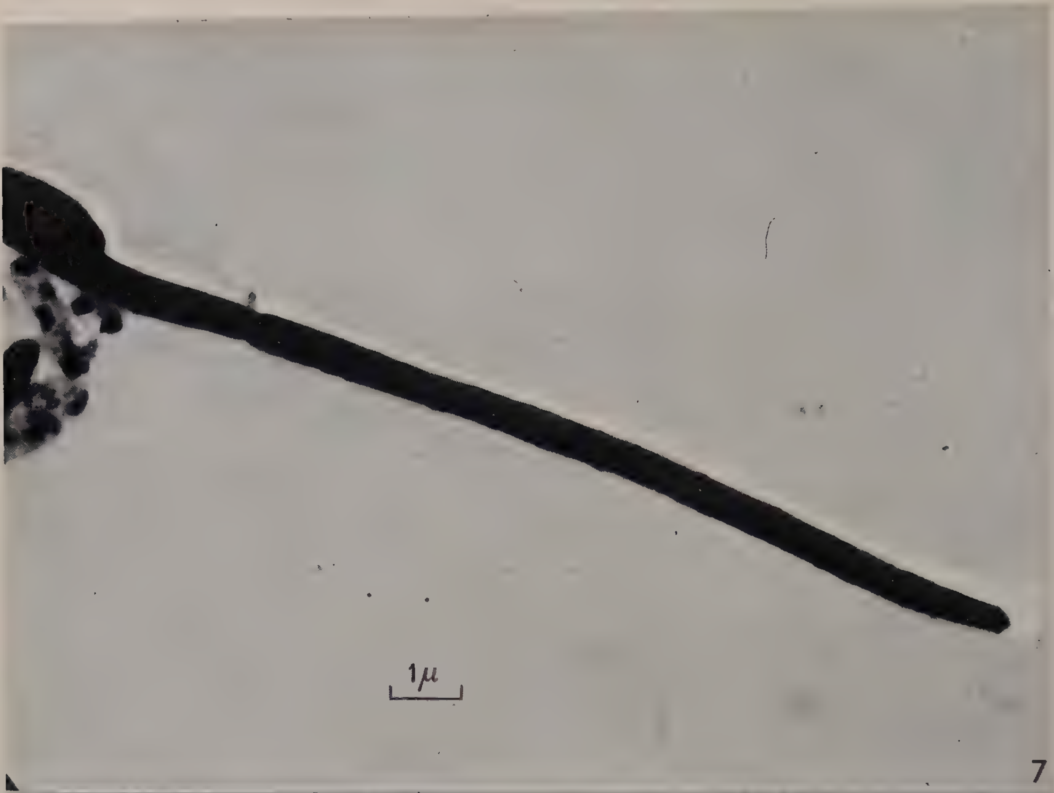
R. C. Valentine & J. R. G. Bradfield—Urea method for counting live bacteria. Plate 1



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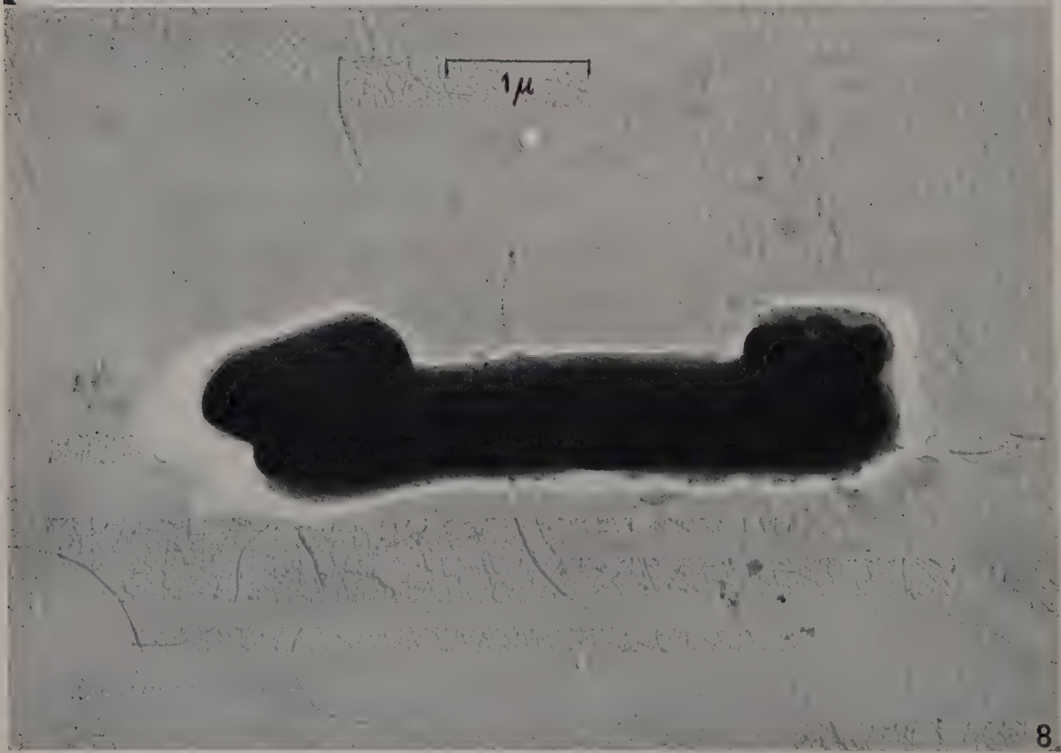

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R. C. Valentine & J. R. G. Bradfield—Urea method for counting live bacteria. Plate 2

EXPLANATION OF PLATES

PLATE 1

The magnification of figs. 1 to 5 is ×5200, shown by a scale equivalent to 1 μ. on Fig. 2. The magnification of fig. 6 is ×8800, also shown by a 1 μ. scale.

Fig. 1. Paracolon bacilli (D. 201 H) taken from an 18 hr. agar slope. Cells in resting phase; few longer than 1·5 μ.

Fig. 2. A microcolony formed by 3·5 hr. incubation of bacilli like those shown in fig. 1 grown directly on an electron microscope specimen grid placed on nutrient agar containing 1 % urea. Cells 1·5–2·3 μ. long.

Fig. 3. The bacilli after incubation for 2·5 hr. on a grid placed on nutrient agar containing 3 % urea. Cells 4–6 μ. long.

Fig. 4. As for fig. 3, but after 6 hr. incubation. Cells 30–50 μ. long.

Fig. 5. Appearance of the bacilli taken from a partly killed culture after 3 hr. incubation on 3 % urea agar. The live cells have grown long and are easily distinguishable from the shorter dead cells. 5*a*, one giant (live) cell and one normal sized (dead) cell. 5*b*, seven giant (live) cells and three normal sized (dead) cells, one of which has lysed.

Fig. 6. Part of a lysed giant cell.

PLATE 2

Fig. 7. *Salmonella paratyphi-C* after 5 hr. incubation on 3 % urea agar. One giant (live) organism 16 μ. long and a clump of normal sized (dead) cells of lengths up to 2 μ., most of which have lysed and contain dense bodies. ×9500 shown by the 1 μ. scale.

Fig. 8. *Proteus morganii* after 5 hr. incubation on 3 % urea agar, showing one long (live) cell of length 4·5 μ. beside two normal sized (dead) cells 1·5 and 1·0 μ. long. The length of the longest cell found on ordinary nutrient agar is 2·0 μ. × 18,800 shown by the 1 μ. scale.

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Mutants of *Chlamydomonas moewusii* with Impaired Motility

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SUMMARY: A method is described for obtaining mutants of *Chlamydomonas moewusii* with impaired motility. Thirteen distinct mutants with paralysed flagella were isolated, and eleven of these were obtained in both mating types by crossing with wild type. No sexual activity was elicited in M. 1060 (*minus*). No allelism was detected among the remaining twelve mutants. In M. 1002 *plus*, the locus for paralysis appears to be very closely linked with that of mating type. In certain mutants, paralysed *plus* gametes, when paired with wild-type *minus*, recovered motility after some hours. Recovery is attributed to the passage of motility factors across the protoplasmic bridge linking the paired gametes. Paralysed *minus* cells may act as donors of such factors when paired with paralysed *plus* cells of an unlike mutant strain. The pairs formed by mating genetically dissimilar gametes are considered as heterokaryons, which can be used to provide information on the identity or non-identity of genes associated with paralysis. The role of a number of transmissible factors in normal flagellar activity is thereby implicated.

In the protist flagellum as in muscle there must be some means by which chemical energy can be converted into movement, and it is likely that a chain of reactions may be involved. Genetic blockage or disturbance at any step will result in impaired motility, and from a study of genetically paralysed mutants we may hope to learn something of the processes controlling the normal functioning of flagella. Many unicellular algae or other flagellates lend themselves well to such studies. Paralysed mutants of *Chlamydomonas moewusii* and *C. dysosmos* have been obtained following ultraviolet irradiation: *C. moewusii* was selected for further study since the species is heterothallic, and thus more suitable for mating experiments and genetic analysis. Normal activity of wild-type cells has already been described (Lewin, 1952*a*).

METHODS

The culture medium contained, per litre: $Ca(NO_3)_2.4H_2O$, 1·0 g.; $MgSO_4.7H_2O$, 0·2 g.; K_2HPO_4, 0·2 g.; traces of Fe, etc. (Lewin, 1952*b*) in glass-distilled water. Solid media were prepared with 1 % Bacto-agar (Difco). When active gamete suspensions were required, the growth medium was supplemented with 1·0 g./l. sodium acetate ($CH_3COONa.2H_2O$) and 1·0 g./l. sodium citrate ($C_6H_5O_7Na_3.2H_2O$), and the procedure outlined by Lewin (1953) followed. All media were sterilized by autoclaving at 15 lb./sq.in. for 15 min. Cultures were grown in constant light (220 f.c.) at controlled temperature (23°).

The origin of certain mutant stocks was described by Lewin (1952*b*). Others were obtained from *plus* or *minus* cells by the following enrichment technique. Cells in 15 ml. of suspension were irradiated with constant stirring for 15 min.

at a distance of 13 cm. from a source of ultraviolet light (Westinghouse 'Sterilamp'). A number of 0·2 ml. samples was then removed and each inoculated into 10 ml. of mineral medium held in a 15 cm. test tube, which also contained a 17 cm. pipette, and which was covered by a glass vial. Tubes were illuminated for 7 days. The pipette was then used to remove cells from the bottom of each culture, in this way decreasing contamination with swimming cells from the upper layers. Samples so removed were streaked on agar plates and incubated in light for 10 days. Since colonies of wild-type cells tended to be more flattened than those of non-motile mutants, the more heaped colonies on each plate were selected, transferred individually to fresh liquid medium, and illuminated for a further 7 days. Mutant cultures could be distinguished from wild type by growth on the bottom of the tube rather than in the surface film. To eliminate duplication, only one clone was selected from each original enrichment tube. Among the mutants so obtained were palmelloid and flagella-less types (cf. Lewin, 1952*b*), which were rejected, as also were variants in which motility was only partially impaired.

To effect recombination between the mutant character and the mating-type locus, paralysed cells were mated by shaking in light with wild-type gametes, zygotes were isolated and germinated singly, and the resulting cell suspensions streaked on agar plates. Progeny clones arising from single colonies were tested for paralysis and mating type, and recombinants were selected, so that wherever possible both *plus* and *minus* clones were obtained for each mutant. Details of the genetic methods employed have been published elsewhere (Lewin, 1953).

RESULTS

General characteristics of mutants

All paralysed mutants conformed more or less to those already described (Lewin, 1952*b*). Swimming activity was impaired, so that cells in liquid cultures accumulated on the floor of the vessel (Pl. 1, fig. 1), but the ability to 'creep' (Lewin, 1952*a*) remained. Colonies on semisolid agar (0·4 %) media were thus capable of spreading, though at a rate considerably lower than that of wild-type (Pl. 1, fig. 2). In some mutants, cells capable of erratic swimming appeared in old cultures: in M. 1011 and M. 1038, rare spontaneous genetic reversions were isolated. Different strains showed small but constant differences in behaviour in cultures of comparable age and treatment. Cells of eight mutants were examined under the electron microscope, but in no case was the disability attributable to any visible structural change in the flagella (Lewin & Meinhart, 1953). So far, all attempts to restore motility by the addition of various substances or cell extracts to the medium have been unsuccessful.

Since the flagella are intimately concerned with the processes of mating (Lewin, 1952*a*), it might be expected that certain paralysed mutants would also be impaired sexually. In fact, paralysed mutants in standing suspensions mated poorly; but the incidence of pairing was considerably improved when mixtures were shaken to ensure frequent flagellar contacts between cells of opposite mating type. M. 475 was exceptional in one respect: whereas the paralysed cells of both mating types paired fairly well with other paralysed mutants, virtually no pairing occurred when this mutant was selfed. Extracts of wild-type cells did not alleviate this self-sterility. M. 1060 (*minus*) appeared completely sterile, producing no pairs even when mixed with wild-type *plus* gametes.

Although M. 1002 *plus* produced sexually active gametes, all attempts to obtain recombination between this paralysis gene and mating type were unsuccessful. Fifteen zygotes were isolated from a cross (M. 1002 *plus* × wild-type *minus*), and from each zygote progeny-clones were tested for paralysis and mating type. In no case was a M. 1002 *minus* or wild-type *plus* strain isolated, indicating very close linkage between the M. 1002 gene and the mating-type locus.

Behaviour of mutants in gamete pairs

When wild-type cells are mated, copulating gametes form pairs which swim for a number of hours before cell fusion takes place, the cells being held together by a narrow bridge of protoplasm (Pl. 1, fig. 3) (Lewin, 1950). Only one gamete, the *plus* partner, is responsible for the propulsion of such pairs: the flagella of the *minus* cell cease to beat once the gamete protoplasts have become joined by a bridge, so that motility of the pair is unidirectional. When wild-type *plus* cells are paired with paralysed *minus*, the pairs are motile; whereas in the reciprocal cross the pairs fail to swim, since the *plus* partner is paralysed.

In well-illuminated cultures, cytogamy occurs after 4–8 hr., but in darkness it is delayed indefinitely. Pairs formed by the mating of wild-type *minus* gametes with *plus* cells of certain paralysed stocks, though initially non-motile, began to swim after incubation for a period in darkness. It could be shown by suitable labelling, e.g. by the use of starved cells of one mating type (Lewin, 1950), that in such cases it was not the *minus* partner which had begun to swim, but the hitherto paralysed *plus* cell. Since recovery was not effected by culture filtrates from wild-type cells, it appeared that the *plus* mutant, paralysed as a result of some genetically controlled cytoplasmic deficiency, was able to receive the missing factor, essential for normal motility, from its non-paralysed *minus* partner. This is illustrated diagrammatically in Fig. 1.

Not all paralysed mutants proved capable of gaining motility when gametes were paired with wild-type cells; while, among those which were capable of recovery, the time required varied considerably. Recovery was more rapid at higher temperatures (e.g. 40°), but all cells lost motility within a few hours at this temperature. Recovery times given in Table 1 were determined for cells maintained at 20° in darkness to suppress cytogamy.

Since the cytoplasts of the two gametes were connected by a protoplasmic bridge, we might regard such pairs as heterokaryons in which genetically unlike nuclei operate in a common cytoplasm. As in fungal heterokaryons, nuclei bearing mutant genes for different physiological impairments could complement one another to produce a wild-type phenocopy. Thus whereas pairs resulting from a selfing cross (e.g. M. 475 × M. 475, or M. 1001 × M. 1001) were never observed to recover motility, the mating of certain dissimilar

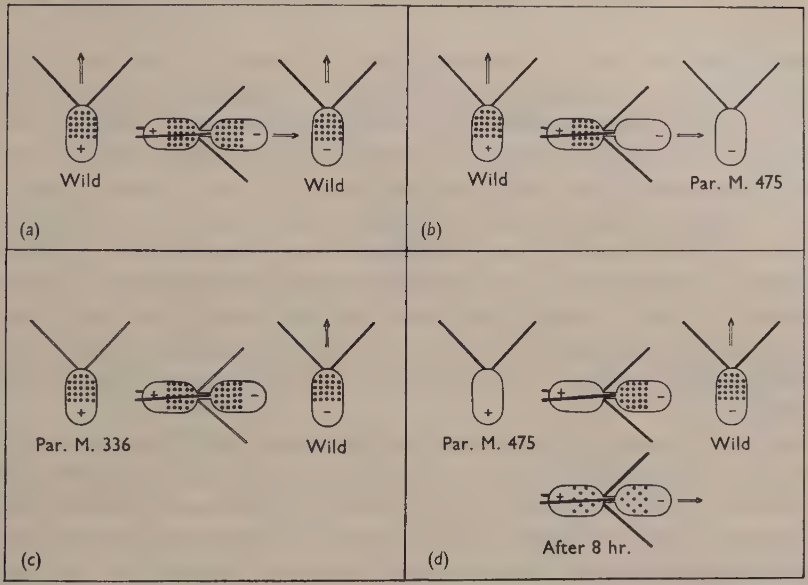

Fig. 1. Diagrams illustrating hypothesis of transmissible motility factors in *Chlamydomonas moewusii*. (*a*) Wild-type *plus* × wild-type *minus*. Pair motile as soon as gametes have copulated; propulsion by *plus* partner. (*b*) Wild-type *plus* × paralysed *minus*. Pair motile as soon as gametes have copulated. (*c*) Paralysed *plus* × wild-type *minus*. Pair remains immotile, since paralysis of M. 336 is not of 'recoverable' type. (*d*) Paralysed *plus* × wild-type *minus*. Pair initially immotile, since *plus* partner genetically paralysed, but becoming normally motile after incubation, after factor initially lacked by *plus* cell has been transmitted from wild-type *minus* partner. (Black spots represent motility factor. White flagella of M. 336 represent paralysis of non-recoverable type. Arrows indicate direction of swimming in motile cells or gamete pairs.)

Table 1. *Times of recovery of* plus *paralysed mutants after pairing with* minus *wild-type gametes*

Mutant	Recovery time (hr.)
M. 1002	2 to 4
M. 475 M. 1001	6 to 8
M. 1017 M. 1021 M. 1022	24 to 48
M. 336* M. 1011 M. 1034 M. 1038 M. 1041 M. 1043	No recovery in 72

* Grown with thiamine (1 mg./l.).

mutants (e.g. M. 475 *plus* × M. 1001 *minus*, or M. 475 *minus* × M. 1001 *plus*) produced motile pairs when gamete mixtures, mated in light, were incubated in darkness.

An attempt was made to separate copulating gametes after the transfer of motility factors into hitherto paralysed cells. A mating suspension (M. 1002 *plus* × M. 1001 *minus*), containing abundant pairs, was incubated in darkness until the pairs, unlike the parental gametes, were actively motile. Samples taken after 3 and 24 hr. were subjected to forty up-and-down passages in a Potter-Elvehjem type homogenizer with a 'Teflon' pestle, resulting in the rupture of 40 % of the pairs, and the organisms were then examined at intervals. Although a large proportion of the gamete pairs remained motile after such treatment, no motile unicells were observed, indicating either that separated *plus* ex-copulants reverted at once to their original paralysed state, or—more probably—that severe cell damage was an invariable concomitant of gamete separation.

Beadle & Coonradt (1944) pointed out that indications of allelism might be obtained by the use of heterokaryon tests of this sort in fungi, though it should be borne in mind that proof of true non-allelism demands genetic analysis. Accordingly, twelve sexually active strains of paralysed *Chlamydomonas moewusii*, independently isolated after ultraviolet irradiation, and all but M. 1002 available in both mating types, were tested by pairing in all possible combinations. Portions of each gamete mixture were maintained in darkness for heterokaryon formation, and in light for zygote production. It was found that *minus* gametes of every mutant strain tested induced recovery of motility in *plus* gametes of 'recoverable' types (see Table 1), except in the cases of selfed pairings. Zygotes from each mating were allowed to germinate in liquid media, and, again in every case except selfings, clones of swimming cells could be isolated from the progeny, indicating that recombination had taken place between different paralysis genes.

DISCUSSION

In a previous study (Lewin, 1952*b*), seven paralysed mutants were obtained after ultraviolet irradiation, even without the use of a special enrichment technique such as that described here, and three of these mutant genes were tested and shown to be genetically non-allelic. It thus appeared that impairment of motility may be a common consequence of mutagenic irradiation, and that a number of different genes may be concerned in the processes of normal flagellar action. The results of the present paper bear this out, and indicate that at least a dozen genes, and probably many more, are involved. Certain of these genes are responsible for the formation of transmissible factors; the heterokaryon tests described above indicate that at least six different factors are implicated. Their nature is at present unknown. We may expect that their ultimate identification will be rendered difficult by the fact that they are apparently not liberated into the medium, and therefore presumably cannot pass freely through the cell membrane in either direction.

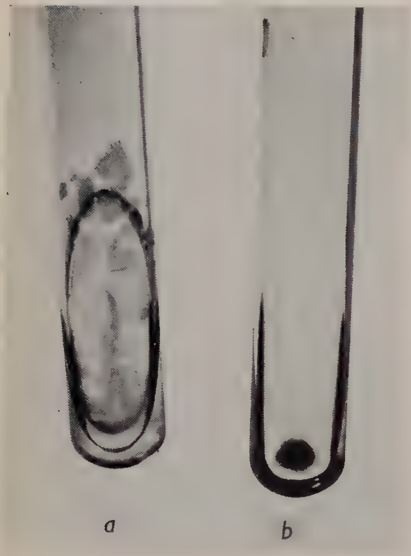

Fig. 1

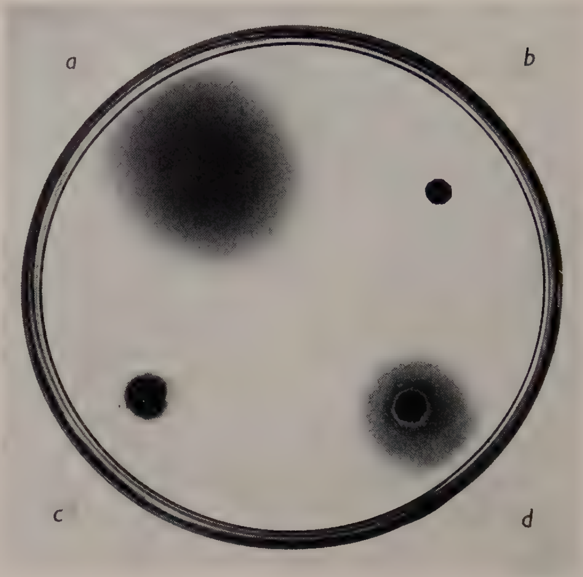

Fig. 2

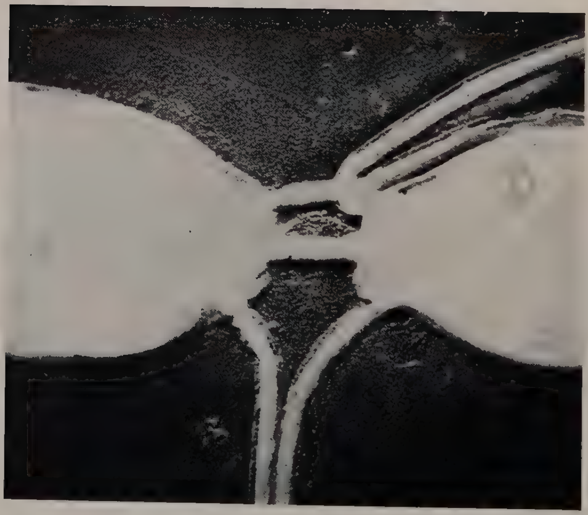

Fig. 3

R. A. Lewin—*Chlamydomonas* with impaired motility

It should be emphasized that in these heterokaryon experiments with *Chlamydomonas moewusii* there was no evidence of genetic transduction, such as that demonstrated in Salmonella by Stocker, Zinder & Lederberg (1953). Analyses of heterozygotes between wild-type and paralysed mutants yielded equal numbers of swimming and non-swimming clones, as expected according to currently accepted genetic theory.

This paper is the fourth in the series: Studies on the flagella of algae. It is issued as N.R.C. no. 1895.

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EXPLANATION OF PLATE

Fig. 1. Sloped liquid cultures in test tubes. (*a*) Wild-type cells tend to accumulate in a surface film, which has here been disrupted by gentle agitation; (*b*) paralysed cells remain on the bottom.

Fig. 2. Spreading colonies on 0·4 % agar, after 2 weeks growth. (*a*) wild-type; (*b*) flagella-less mutant; (*c* and *d*) paralysed mutants (M. 1017 and M. 475).

Fig. 3. Electron micrograph of gamete pair, showing bridge between anterior ends of copulating cells (× 10,000).

Photographs 1 and 2 by H. F. Roberts.

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Nuclear Segregation and the Growth of Clones of Spontaneous Mutants of Bacteria

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SUMMARY: An investigation of the rate of increase of spontaneous mutants and the subsequent increase in mutant proportion in a bacterial culture revealed discrepancies between the observed results and those expected on the assumption that mutant and parent grew with equal rates. These discrepancies could not be accounted for in terms of a selective difference between established mutants and their parents since, when the two were mixed together in reconstruction experiments, they fared equally well for hundreds of generations. Rather the discrepancies indicated a difference between parents and *new* mutants. The data were consistent with the hypothesis that the mutation occurred independently among one of four mutable units (nuclei) and that the mutant nucleus was 'dominant' over its sister non-mutant nuclei in the heterocaryon so formed. As a consequence, a delay of two generations ensued before the mutant unit segregated into the homocaryotic ancestor of a mutant clone. This process delayed the onset of an increase in mutant numbers after mutation. The accurate prediction of the pseudo-equilibrium level of mutants, based on verifiable assumptions of periodic selection, mutation and segregation lag, is added evidence for the occurrence of a two-generation delay before the increase of the mutant clone. This phenomenon, called segregation lag, is a source of error in the calculation of mutation rate by methods involving the numbers of mutants found in bacterial cultures. Furthermore, because bacteria may be multinucleate, the rate of mutation/bacterium/generation is not the same as the rate of mutation/mutable unit (nucleus)/generation.

That a growing bacterium may contain more than one body consisting of deoxyribonucleic acid is well known. Witkin (1951) was the first, however, to offer direct evidence indicating that these bodies contain genetic material and are the counterparts of nuclei. Her evidence was threefold. First, the ratio of wholly mutant colonies to those containing mutant sectors was proportional to the ratio of uninucleate bacteria in the irradiated culture which gave rise to the colonies. This finding is consistent with the hypothesis that sectored colonies originate through segregation of the nuclear bodies during growth. Secondly, there was a proportionality between the frequency of induced mutants, whole and sectored, and the frequency of bacteria with more than one nuclear body. Finally, there was some correlation between the types of sectored colonies and the most frequent number of nuclear bodies/multinucleate cell; cultures with mostly binucleate bacteria gave rise predominantly to half sectors while cultures consisting for the most part of cells with four nuclei gave rise predominantly to quarter sectors. A more extensive correlation was not attempted because of the difficulty in determining nuclear numbers.

This difficulty is even greater with bacteria that have been grown on chemically defined media. Yet it is sometimes necessary to know the average number of similar units capable of independent mutation in bacteria growing under such conditions. Consider the case where a mutation occurs in one of two identical units within a bacterium. If the mutant character were recessive it would not express itself until that bacterium had divided and given rise to two daughter cells, one homocaryotic for mutant and the other for non-mutant units. The mutation would *seem* to have occurred in the mutant daughter and hence the mutation rate would be underestimated by a factor of one half. If the mutant character were dominant, and there were no delay in the physiological development of the phenotype (no phenotypic lag), the first bacterium in which the mutation had occurred would express the mutant character. After the first division the number of mutants in that clone would not yet have increased because of the segregation of mutant from non-mutant units. Only after the homocaryotic mutant daughter had divided would the number of mutants increase (Fig. 1). This segregation lag acts like selection against the mutants inasmuch as it depresses their rate of increase in a culture. It is thus a source of error in calculating mutation rate, mutant distributions and the kinetics of population change.

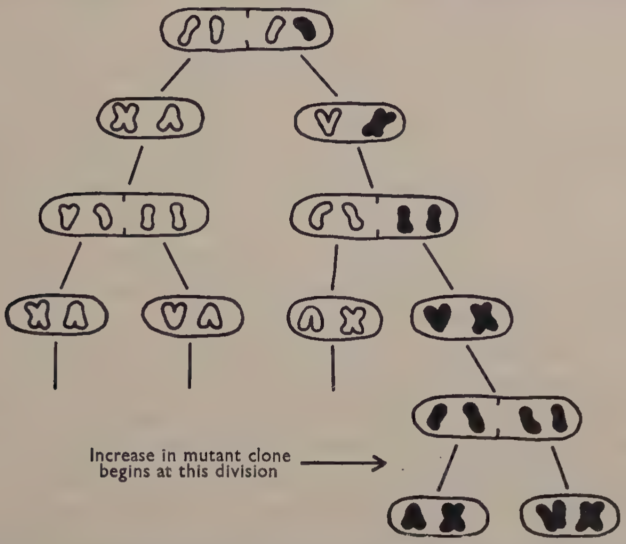

Fig. 1. Diagram of the segregation of a mutant nucleus from its non-mutant sister nuclei in a heterocaryotic bacterial cell. The assumptions are that: (1) there are four nuclear bodies, with regular segregation and thus 2 generations of segregation lag; (2) the mutation is 'dominant' in heterocaryons; (3) there is no phenotypic delay. Thus, throughout the first two generations there is but one mutant present in the clone. The increase in the mutant clone begins at the third division.

Where segregation lag is in operation, the population of mutants will increase as though they were at a selective disadvantage, but when mutant and parent are put in competition in artificial mixtures the mutant need not be overgrown. Such a situation has been found with a histidine-requiring (h^-) strain and its independent (h^+) mutant. No selective differences could be observed between them in extensive tests. Yet the increase in numbers of h^+

mutants was depressed below that expected from experimentally determined mutation rates and the assumption of no selective differences.

This paper will deal with experiments designed to reveal and measure the extent of segregation lag in this system and hence the average number of mutable units in these bacteria. The information obtained will be related to the problems of making a reliable estimate of the mutation rate and of developing a correct expression for the dynamics of population change.

MATERIALS AND METHODS

The parent strain used in these experiments was a histidine-requiring mutant (h^-) of *Escherichia coli* (*Bacterium coli*), strain 15. The manner of cultivating it, the composition of the chemically defined medium and other technical details are described elsewhere (Ryan & Schneider, 1948). Unless otherwise indicated, the glucose content of the medium was 0·5 % (w/v) and the cultures were unshaken during growth. The h^- bacteria mutate to a histidine independent state (h^+), and the numbers of h^+ mutants can be determined by plating cultures, washed in 0·9 % (w/v) saline, into agar devoid of histidine where the h^- parents do not grow. That this procedure reliably measures the number of h^+ organisms in the culture plated has been demonstrated elsewhere (Ryan & Schneider, 1949). That washing by centrifugation and resuspension resulted in no significant loss of bacteria was shown in eleven experiments where plating washed cultures yielded 98 % of the number of colonies found upon plating unwashed cultures, with the total numbers of bacteria varying between $3{\cdot}1 \times 10^2$ and $7{\cdot}3 \times 10^8$/ml. Platings of both h^+ and h^- bacteria, always done in duplicate, were made with saline dilutions designed to yield *c.* 50 h^+ or h^- colonies/plate. Under such conditions the variance of numbers was approximately equal to the mean as would be expected if the only important error was that introduced by random sampling.

The h^+ mutants produced in these experiments formed a homogeneous group. Not only did they all grow at the same rate and to the same extent, but, unlike some growth-factor-independent mutants (Davis, 1950), they did not vary significantly in the amount of histidine they produced. As a matter of fact, they did not excrete appreciable amounts into the medium, since only extremely rarely were satellite colonies or haloes of growth formed by h^- bacteria crowded around the mutant h^+ colonies. These h^+ mutants were probably not due to a mixture of suppressor- and true back-mutations (Witkin & Kennedy, 1951) because Lieb (1951) showed, by studying nineteen randomly isolated h^+ reversions of independent origin, that their rates of mutation to the h^- state were the same and averaged $1{\cdot}2 \times 10^{-6}$/bacterium/generation. Although mutability is an extremely sensitive test, a critical decision cannot be made since, despite repeated attempts to introduce F^+ factor (Cavalli, Lederberg & Lederberg, 1953) into strain 15, sexual crosses have been unsuccessful.

In some experiments organisms were employed which carried an additional genetic marker lac^-, one which prevented the fermentation of lactose. A marked strain (h^-lac^-) was obtained after exposure to ultraviolet light of h^- bacteria on the surface of Difco-Endo agar plates until a survival of *c.* 10^{-6} was obtained. From the h^-lac^- stock, 10 independent h^+lac^- strains were secured after plating into medium devoid of histidine. When h^+lac^- bacteria were to be added, for example, to a culture of their h^-lac^+ progenitor, all 10 of the h^+lac^- strains were separately grown and then mixed together in equal proportions. A sample of this mixture was added to the h^-lac^+ culture. Similarly, 10 cultures of independently mutated h^+lac^+ were used for additions to h^-lac^- bacteria and, when fitter-type h^- and h^+ bacteria were isolated after serial transfer, 10 stocks of each were established and all 10 used whenever possible.

Diagnosis of the proportion of lac^- bacteria in a mixed culture was made in early experiments by streaking from colonies on to the surfaces of Difco-Endo or eosin-methylene blue indicator plates where lac^- bacteria formed uncoloured colonies in contrast to lac^+ bacteria which formed purple colonies. In later experiments the replication technique of Lederberg & Lederberg (1952) was employed.

The neutral character of the stable 'lac' markers was shown in a number of ways. The h^-lac^+, h^-lac^-, h^+lac^+ and h^+lac^- strains, when grown separately on medium containing histidine, had indistinguishable rates and final levels of growth, and the h^- stocks contained the same numbers of h^+ mutants. When, in four experiments, mixtures of h^+lac^- and h^+lac^+, with an average initial ratio of 63:37, were grown for 232 generations the final average proportion was 72:28. It can be concluded that the introduction of the lac marker did not affect to any great extent the selective qualities of the strain. Further evidence establishing this point will be presented below.

All strains used in these experiments were purified by at least two repeated isolations from Difco-Endo agar. Samples of the stocks were then spread on surfaces of nutrient agar containing 0·9 % (w/v) NaCl where their sensitivity to the seven T-coliphages was tested. All, like the parent strain, were sensitive only to phage T_2.

In those cases where cultures were serially transferred the procedure was as follows. Growth was allowed to occur in 125 ml. Erlenmeyer flasks containing 50 ml. basal medium supplemented with 25 μg. L-histidine monohydrochloride/ml. Unless otherwise mentioned, the inoculum consisted of 0·5 ml. of such cultures which had entered the stationary phase at 37° and were between 8 and 16 hr. old. Such an inoculum contained *c.* $2{\cdot}5 \times 10^8$ h^- bacteria which, during 6 hr. of growth in 50 ml. of fresh medium, produced *c.* $2{\cdot}5 \times 10^{10}$ progeny in 6·64 generations. It was necessary to employ such a large inoculum because the frequency of h^+ bacteria at the beginning of the experiments was as low as 10^{-7}. In such an event 0·5 ml. contained *c.* 25 h^+ bacteria, a number which is not likely to be subject to drastic distortion by sampling error. Gradually the number of h^+ organisms in the inocula became much greater, in some cases being as large as 1000. It is felt that adequate samples of the previous population were taken for inoculation into the next.

The optical density of cultures in the stationary phase of growth remained constant for a matter of many days. The gradual death of bacteria began after the first day, but until that time the viable number was equal to the total number as determined with a Petroff-Hauser counting chamber; for example, in two determinations made after the bacteria were in the stationary phase for 12 hr., the viable number was $5{\cdot}2\times10^{8}$/ml. while the total number was $4{\cdot}8\times10^{8}$/ml. During the period within which samples were taken routinely for inoculation, there was no detectable shift in the proportions of h^{+} and h^{-} bacteria (Table 1).

Table 1. *Proportion of* h^{+} *bacteria during the early part of the stationary phase of growth*

The proportion of h^{+} bacteria was varied in the last three experiments by introducing extra h^{+} organisms with the inoculum.

Expt.	Age of culture (hr.)	No. of h^{-} bacteria per ml.	No. of h^{+} bacteria per ml.	Frequency of h^{+} bacteria
1	8·0	$5{\cdot}3\times10^{8}$	$1{\cdot}2\times10^{2}$	$2{\cdot}3\times10^{-7}$
	12	$5{\cdot}6\times10^{8}$	$1{\cdot}1\times10^{2}$	$2{\cdot}0\times10^{-7}$
	16	$5{\cdot}0\times10^{8}$	$9{\cdot}9\times10^{1}$	$2{\cdot}0\times10^{-7}$
2	0	$1{\cdot}0\times10^{7}$	4·7	$4{\cdot}7\times10^{-7}$
	6·0	$2{\cdot}3\times10^{8}$	$1{\cdot}2\times10^{2}$	$5{\cdot}2\times10^{-7}$
	12	$5{\cdot}1\times10^{8}$	$2{\cdot}6\times10^{2}$	$5{\cdot}1\times10^{-7}$
3	12	$5{\cdot}8\times10^{8}$	$1{\cdot}7\times10^{3}$	$2{\cdot}9\times10^{-6}$
	18	$5{\cdot}4\times10^{8}$	$1{\cdot}7\times10^{3}$	$3{\cdot}2\times10^{-6}$
4	9·7	$4{\cdot}5\times10^{8}$	$4{\cdot}5\times10^{3}$	$1{\cdot}0\times10^{-5}$
	12	$4{\cdot}5\times10^{8}$	$4{\cdot}6\times10^{3}$	$1{\cdot}0\times10^{-5}$
	16	$4{\cdot}1\times10^{8}$	$3{\cdot}9\times10^{3}$	$9{\cdot}5\times10^{-4}$

Initial increase of h^{+} *bacteria in* h^{-} *cultures*

In order to determine the rate at which h^{+} bacteria increased in number after they first arose by mutation in h^{-} cultures, *c.* 50 h^{-} bacteria were inoculated into a flask containing 50 ml. basal medium + histidine. At various times after inoculation, samples, varying in different experiments from 5 to 12 ml. were removed and the numbers of h^{+} and h^{-} bacteria determined by differential plating. The estimated total numbers of bacteria present at different times are shown in Fig. 2. The data were collected from fourteen different experiments and the individual growth curves of the h^{-} bacteria showed lag periods (defined as the time at which the straight line describing logarithmic growth intercepts the level of inoculum size) varying between 0 and 5 hr. That the logarithmic rates of growth were the same is shown by the goodness of fit when the lag periods were equated to the average of 3·75 hr. as was done in the construction of Fig. 2. Even after a corresponding adjustment, the individual curves for the h^{+} bacteria intercepted the level of one bacterium at times varying from 15·5 to 20 hr. This variation is to be expected if mutations occur at random, for in some cultures the first mutation takes place earlier than in others. In order to portray the average rate of increase of the h^{+} bacteria, their individual curves were equated at the level of 10^{3} bacteria with the average curve. It will be observed that the h^{-} bacteria grew to a final population of $2{\cdot}5\times10^{10}$ organisms, the generation time during the logarithmic phase being about 50 min. The h^{+} bacteria increased at a constant rate which cannot be distinguished from that of the h^{-} bacteria.

This fact was surprising inasmuch as the total increase in h^{+} bacteria, assuming a constant mutation rate and no selection for or against the mutant,

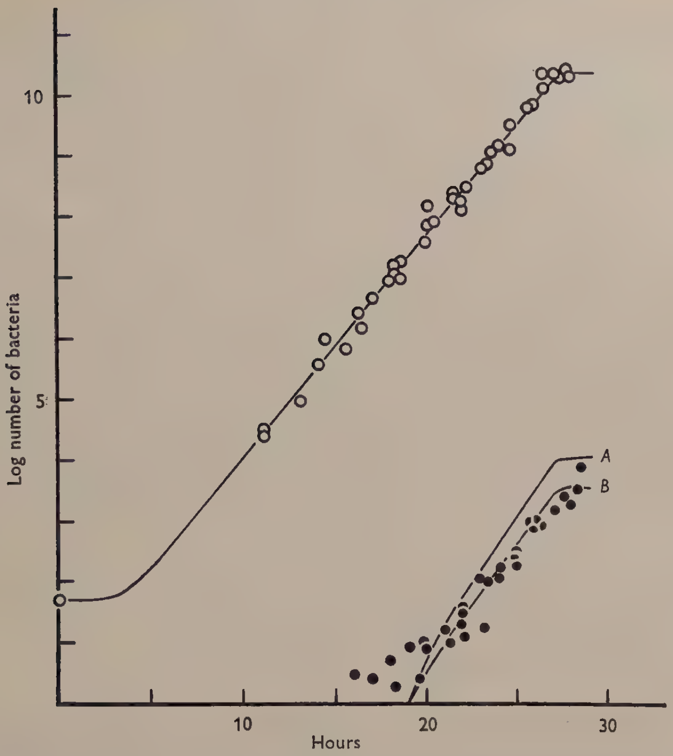

Fig. 2. The rate of increase in the number of h^- bacteria (open circles) and the h^+ mutants (black circles) arising among them as determined by sampling suspensions of cultures which contained histidine and had been inoculated with a small number of h^- organisms. The ordinate measures the $\log_{10}$ of the number of bacteria; the abscissa, the number of hours elapsing between inoculation and sampling. The text describes how the data were used to construct this figure. Curve *A* through the numbers of h^+ bacteria (black circles) describes the behaviour expected on the assumption of no segregation lag. The curve *B* through the same points is that expected if segregation lag lasts for two generations.

should follow the series 1, 4, 12, 32, ..., if the following model be valid. Assume that, on the average, when there are n parental h^- bacteria, the first mutation occurs. When the parents have undergone a division, so has the mutant; but among the parents, now double in number, twice as many mutations occur on the average. The increase of parents and mutants would then take place as follows:

Generation	Parents	Mutants: New Old	Total number of mutants
1	n	1	1
		↘	
2	$2n$	2+2	4
		↘ ↘	
3	$4n$	4+4+4	12
		↘ ↘ ↘	
4	$8n$	8+8+8+8	32
–	–	– – – –	–

The new mutants have been added at each generation to the left column of the triangle and their progeny are carried forward as the arrows indicate. Other models are possible (Armitage, 1953; Kendall, 1953) but all call for a greater than logarithmic increase in the number of mutants. Yet the actual increase seems to be nearly logarithmic; curve *A*, which does not fit the points in Fig. 2, is that calculated from the above model, where the number of mutants at any generation, g, is equal, on the average, to $g \times 2^{g-1}$.

If, after the first mutant appeared, no further mutations occurred, a logarithmic series would be obtained. This hypothesis has been tested by determining the proportions of h$^+$ mutants that carry parental markers in a mixture of h$^-$lac$^+$ and h$^-$lac$^-$ bacteria. About 50 organisms of each strain were either inoculated together into 50 ml. defined medium + histidine, or the h$^-$lac$^+$ bacteria were inoculated 1 hr. after the h$^-$lac$^-$. Samples of the mixed cultures were taken at different times and characterized for their h$^-$ and h$^+$ content. The results shown in Table 2 are averages of two experiments and each percentage is based upon the isolation and characterization of at least 40 colonies. The occurrence of a mutation in one h$^-$ strain neither prevented the occurrence of mutations in the other nor seemed to change at all the numbers of mutants produced by the second strain. This evidence is inconsistent with the hypothesis that the logarithmic increase in number of h$^+$ bacteria is due to interference with subsequent mutation.

Table 2. *The lack of influence of one mutation on the occurrence of others*

Age of culture (hr.)	No. of h$^-$ bacteria per ml.	No. of h$^+$ bacteria per ml.	Frequency of h$^+$ bacteria	Percentage of lac$^+$ bacteria	
				among h$^-$ bacteria	among h$^+$ bacteria
		h$^-$lac$^-$ inoculated with h$^-$lac$^+$			
20	$2{\cdot}3 \times 10^7$	3·1	$1{\cdot}3 \times 10^{-7}$	59·0	69·3
23	$2{\cdot}2 \times 10^8$	$1{\cdot}8 \times 10^1$	$8{\cdot}2 \times 10^{-8}$	72·7	72·0
27	$5{\cdot}2 \times 10^8$	$5{\cdot}9 \times 10^1$	$1{\cdot}1 \times 10^{-7}$	73·3	67·0
		h$^-$lac$^-$ inoculated one hour before h$^-$lac$^+$			
20	$1{\cdot}8 \times 10^7$	1·7	$9{\cdot}4 \times 10^{-8}$	28·0	25·4
23	$1{\cdot}1 \times 10^8$	$1{\cdot}4 \times 10^1$	$1{\cdot}3 \times 10^{-7}$	36·0	18·0
27	$5{\cdot}2 \times 10^8$	$6{\cdot}8 \times 10^1$	$1{\cdot}3 \times 10^{-7}$	30·6	36·0

On the other hand, if each mutant after its appearance survived but did not divide, or divided very slowly, an increase series of 1, 3, 7, 15, ..., $2^g - 1$, which is almost logarithmic, would be obtained. This hypothesis of almost complete selection against h$^+$ mutants was tested by reconstruction experiments in which known numbers of h$^+$ bacteria were added to h$^-$ cultures. Cultures of h$^-$ bacteria, begun with an inoculum of *c.* 50 organisms and serially transferred, have a proportion of *c.* 10^{-7} h$^+$ organisms immediately after the first mutation; the proportion then rises to a pseudo-equilibrium of *c.* 10^{-6} which is achieved after more than 100 generations (Atwood, Schneider & Ryan, 1951). As a consequence simple reconstruction experiments can be interpreted only when h$^+$ bacteria are added in numbers large enough to escape significant additions by the mutations occurring among the h$^-$ bacteria. In order to be able to study selection at ratios of h^+ to h^- bacteria approximating those existing when mutations first occur advantage was taken of the lac marker. Thus, h^+lac^- bacteria were added to cultures of h^-lac^+ organisms in various proportions, the mixtures were serially transferred and their composition determined at intervals. In order to ascertain a possible influence of the lac^+-lac^- difference on the outcome of these experiments the reciprocal mixture, h^+lac^+ in h^-lac^-, was studied.

The position of the lac marker was of no consequence and no mixture exhibited any selective difference between h^+ and h^- bacteria. Thus in 19 experiments, with markers in both combinations and where the initial h^+ proportions were at various levels between 10^{-4} and 10^{-6}, the proportions after 166 generations of growth were, on the average, 96·6 % of those at the start. In 16 experiments, where the initial h^+ proportion was *c.* 10^{-7}, the proportion after 60 generations was found to be, on the average, 104 % of that at the beginning. Again in 5 experiments, where the initial proportions were between 46 and 62 %, the proportions after between 28 and 133 generations changed by an average factor of 0·952.

Thus, h^- and h^+ organisms fared equally well in competition. After some 200 generations, however, a fitter-type mutant appeared, usually in the preponderant component of the mixture, and eliminated all other types present. An analysis of this independent phenomenon is presented elsewhere (Atwood *et al.* 1951). It is of interest here only to note that for more than a hundred generations selective difference between h^+ and h^- bacteria cannot be observed. Hence, drastic selection cannot explain the discrepancy shown in Fig. 2 unless there are differences between new mutants, whose behaviour the figure portrays, and established mutants of the sort used in the reconstruction experiments.

One difference between new and old mutants lies in the fact that the former would be heterocaryotic if the mutation occurred independently and in only one of the several nuclei present. Bacteria from established mutant cultures would be homocaryotic for mutant nuclei since the segregation shown in Fig. 1 would already have taken place during the initiation of the colony from which the mutant clone was first isolated. The activity of growth-factor-independent mutants in heterozygotes (Lederberg, 1949) and the fact that they probably occur as zero-point mutants (Ryan, 1954; Ryan, Fried & Schwartz, 1954) would indicate that the h^+ condition is dominant. If this were true and the development of the h^+ phenotype required less than a generation, then the lag in the increase of the mutant clone, imposed by the necessity for the segregation of mutant from non-mutant nuclei, would have the following effect on the rise of mutant number. Assume that each bacterium has each genetic unit represented 4 times and that mutations occur rarely and at random among them. A two-generation segregation delay ($\Delta=2$) would result (Fig. 1), and the effect on the model discussed above would be as shown on p. 372. This series, which the formulation $2^{g-3}(g+4)$ describes for all but the first generation, is very close to logarithmic and could fit the data in Fig. 2 (curve *B*).

Generation	Parents	Mutants New Old	Total number of mutants
1	n	1	1
		↘	
2	$2n$	2+1	3
		↘ ↘	
3	$4n$	4+2+1	7
		↘ ↘ ↘	
4	$8n$	8+4+2+2	16
–	–	– – – –	–

The non-segregation-lag model also predicts an almost logarithmic rate of increase of h^+ mutants after their number in the culture becomes large. The most sensitive portion of the mutant increase curve for a discrimination between the two models is the low region. Yet it is just here that the data in Fig. 2 are least accurate. The data for numbers of h^+ bacteria less than 30 are based on counts of 1 to 6 bacteria in the samples. Such counts are inaccurate in themselves and, furthermore, although mutation on the plates does not occur to a great extent when washed agar is used, the occurrence of only one or two plate mutations would profoundly distort counts of such small numbers of h^+ bacteria. For this reason data were collected by another method which did not involve the use of agar. At different times samples were taken from a 50 ml. culture of h^- bacteria, washed, resuspended in defined medium devoid of histidine and distributed, with or without dilution, in a series of 1 ml. samples. If any of these samples contained one or more h^+ bacteria, visible turbidity resulted from their growth; if no h^+ bacteria were introduced, no turbidity resulted, for the h^- bacteria present did not grow in the absence of histidine.* By the use of the fraction of the cultures which did not contain h^+ bacteria and the Poisson formulation, it was possible to calculate the average number of h^+ bacteria and from that, the total number that were distributed. In this way errors resulting from mutation on the plates were avoided.

In order to circumvent errors implicit in the sampling of small numbers of mutants the entire contents of whole cultures would have to be assayed. But, because in different cultures the first and succeeding mutations may occur at different times, for every sampling time a large number of cultures would have to be assayed. One might then suppose that the average number of mutants found could be used to construct an increase curve. This procedure is, however, invalid on theoretical grounds. The individual populations of the large number of cultures form in reality one large population containing a number of mutants equal to the number of cultures multiplied by the average number of mutants/culture. As was previously pointed out, when the mutant number is large the expected curves, whatever the hypothesis, all approximate to a logarithmic increase; discrimination between experimental and theoretical curves must be made in regions where the number of mutants is very small. The value of the average number of mutants is, furthermore, seriously affected by chance early mutations contributing disproportionately large bursts of mutants. The *median* number of mutants is, on the other hand, not so affected. Fortunately, through the efforts of Lea & Coulson (1949), the median of the numbers of mutants in a series of different cultures has been calculated for

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* In such stationary cultures of h^- bacteria the h^+ mutants contained grow to visible turbidity in about 24 hr. at 37° as was shown by the introduction of single marked h^+ bacteria. Nevertheless, for a period of about 10 days adaptive overgrowths continued to occur with low frequency in stationary h^- cultures. It was possible to show that these late appearing adaptations were due to h^+ bacteria which were not present at the time of distribution but which appeared later. Perhaps they represent mutations of h^- bacteria in the stationary phase but they may also be due to release, during the stationary phase, of genotypically h^+ bacteria from a phenotypic lag. Evidence secured to make a distinction between these possibilities will be presented elsewhere. In connexion with Fig. 3, however, it should be noted that these stationary phase adaptations were not counted, since observation was limited to the 24 hr. period required for the overgrowth of h^+ bacteria originally present in the sample.

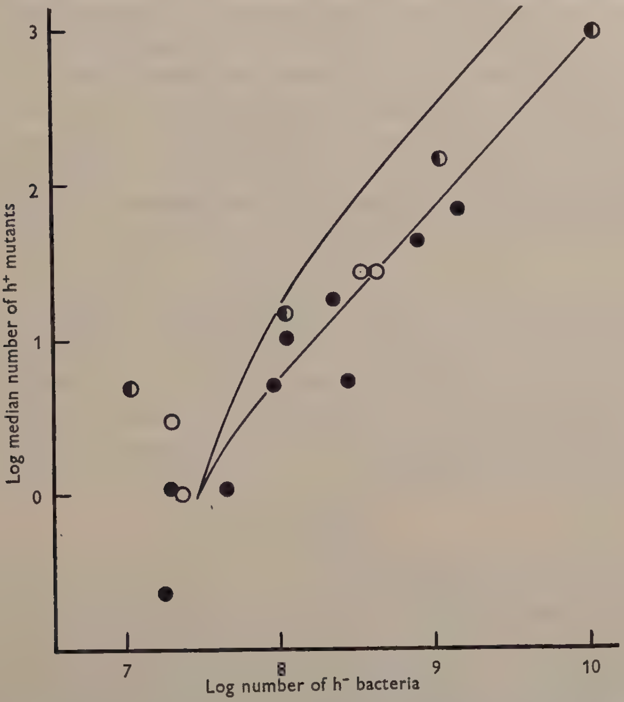

Fig. 3. The rate of increase in the median number of h$^+$ mutants among a series of similar cultures of spontaneously mutating h$^-$ bacteria. The data presented as black circles came from three different experiments in which the whole contents of each culture were assayed by the method of fractionation in liquid medium described in the text. In two of these experiments the bacteria were washed in a refrigerated centrifuge to prevent all growth from the moment of sampling. The data presented as open circles came from two experiments in which at a given time a series of 2 ml. suspensions, washed in the cold, were plated whole along with three saline rinsings. The data presented as half circles are the medians of the values found in the fourteen experiments summarized in Fig. 2. Each point is the median of between 10 and 18 cultures. The ordinate measures the $\log_{10}$ of the median number of h$^+$ mutants; the abscissa, the $\log_{10}$ of the average number of h$^-$ bacteria in the series of cultures assayed. The upper curve is theoretical (see text); the lower was drawn by eye to fit the experimental points.

different average numbers of mutations on the assumptions of random mutation and equal growth of mutant and parent. Thus a theoretical curve for the increase of the median numbers of mutants can be compared with that observed for a large number of cultures whose entire contents were assayed at different times.

Fig. 3 represents such a comparison. Clearly the theoretical curve, constructed on the assumption that the mutants increase in number immediately without a segregation lag, does not fit the data. The equations for the distribution of the numbers of mutants among replicate cultures, where the mutants are assumed to have a delay in the onset of growth, have not been formulated and solved. As a consequence, although the data deviate from the theoretical curve in the fashion expected for mutants undergoing segregation lag, it cannot be said whether they are consistent with the operation of a segregation lag of any given number of generations; the data are simply inconsistent with the assumption of no segregation lag.

Increase in h^+ proportion during serial transfer

An approach to the question of the length of the lag due to nuclear segregation can be made by the mutation rate calculation first used by Kaplan (1947) and by Stocker (1949). Consider a population of size N in which no mutations have yet occurred. If the chance of mutation/bacterium/generation is a, when the population has increased to $2N$ the number of mutants will be $2Na$ and the proportion of mutants to parents

$$\frac{2Na}{2N} = 1a \tag{1}$$

(a is considered to be small so that $2N$ is not greatly different from $2N-2Na$; in which event back mutation, if it also occurs slowly, can be neglected). When $4N$ parents are produced at the next generation, $4Na$ new mutants will appear on the average and the old mutants will have doubled to $4Na$. The mutant proportion will then be approximately

$$\frac{4Na+4Na}{4N} = 2a. \tag{2}$$

At the next generation the proportion of mutants will be $3a$, and so on according to the relation

$$\frac{\text{Mutants}}{\text{Parents}} = ga, \tag{3}$$

where g is the number of generations.

When, however, segregation lag is in operation, the rate of increase in the proportion of mutants will be slower. If we assume that segregation lag lasts for one generation, involving the separation of two nuclei, then the increase can be calculated as follows: when the number of parents is $2N$ the proportion of mutants is

$$\frac{2Na}{2N} = 1a. \tag{4}$$

At the next generation the number of new mutants will be $4Na$ but the division of old mutants will have given rise to only $2Na$ mutant progeny. The proportion of mutants would therefore be

$$\frac{4Na+2Na}{4N}=1{\cdot}5a. \tag{5}$$

After another generation the first lot of mutants will have passed through their lag and doubled to $4Na$; the second crop, still in segregation lag, will have divided without increasing the mutant number and will, therefore, contribute only $4Na$; $8Na$ new mutants will have appeared through mutation. The mutant proportion will, therefore, be

$$\frac{4Na+4Na+8Na}{8N}=2a. \tag{6}$$

The general formula for increase in proportion after the first generation is

$$\frac{\text{Mutants}}{\text{Parents}}=\frac{ga}{2}+C_1, \tag{7}$$

where C_1 is a constant whose magnitude can be determined graphically. The denominator is always the average number of nuclei being segregated which is related to the number of generations of segregation lag, Δ, by the expression 2^{Δ}.

When the segregation of four nuclei is involved and lasts 2 generations the relation for the increase beyond 2 generations is

$$\frac{\text{Mutants}}{\text{Parents}}=\frac{ga}{4}+C_2. \tag{8}$$

In order to determine the actual rate of increase in the proportion of h^+ mutants, cultures of h^- bacteria were serially transferred in the presence of histidine and allowed to accumulate h^+ mutants. This accumulation involved a rise in h^+ proportion from 10^{-7} to 10^{-6} in the course of some 150 generations. After that time the mutant proportion is maintained at a relatively constant level by the operation of periodic selection (Atwood *et al.* 1951). Fitter-type h^- bacteria had been isolated during the course of such pseudo-equilibrium which had persisted for thousands of generations through serial transfer. The more advanced of these fitter-type h^- organisms, when used to establish a new line of serial transfer, showed increases in h^+ proportion that overshot the pseudo-equilibrium level characteristic of the original h^- strain. Since the period of increase in the proportion of h^+ mutants was longer in cultures of fitter-type h^-, it was possible to measure the rate of increase more accurately. Fig. 4 shows, as an example, data for the increase in h^+ mutants in fitter-type strain $h^-\alpha$. The slope is linear and equal to the rate of mutation/mutable unit/generation or to a, $\frac{1}{2}a$ or $\frac{1}{4}a$ according to the length of the segregation lag involved. Table 3 shows a summary of the results obtained with several strains where the average rate of increase in h^+ proportion was $0{\cdot}81\times10^{-8}$.

At the beginning of each of the serial transfer experiments the rate of mutation of the h^- strain under consideration was determined by the Poisson method. This involved distributing to separate tubes 2 ml. samples of medium which had been so inoculated that each aliquot received *c.* 50 h^- bacteria. The medium contained only 0·125 μg. histidine/ml. which limited the total amount of growth to *c.* 2×10^7 h^- bacteria. This level is just at the limit of visibility. The h^+ mutants, on the other hand, were not restricted by the limiting amount

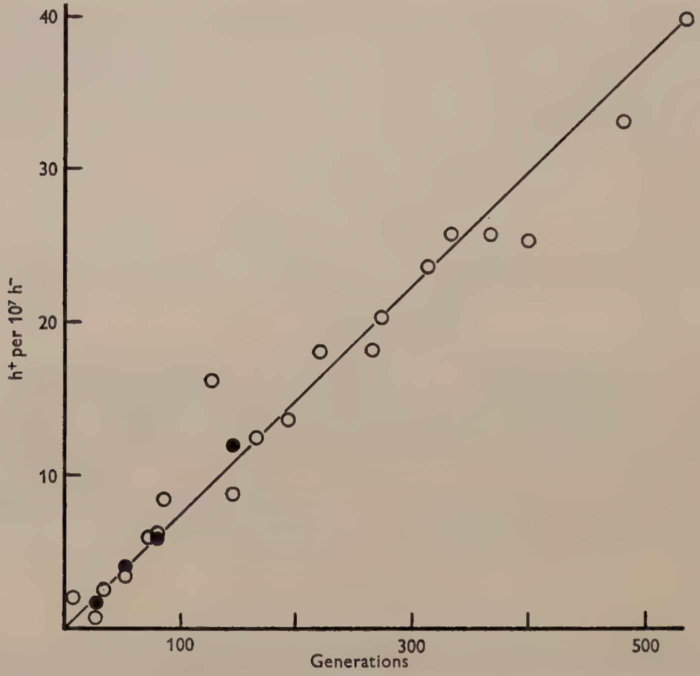

Fig. 4. The proportion of h^+ mutants arising during the serial transfer of fitter-type strain h^-_α (open circles) in medium containing histidine. The ordinate measures the number of h^+ mutants/10^7 h^- bacteria; the abscissa, the number of generations of growth during serial transfer. The results were obtained from two separate experiments. The black circles describe the increase in the original h^- strain of the h^+ proportion during the period of linearity before an equilibrium is achieved.

Table 3. *Rates of mutation from h^- to h^+ determined by two methods*

Strain	Rate of increase of h^+ proportion ($\times 10^{-8}$)	$\times^4$ ($\times 10^{-8}$)	Rate of mutation by Poisson method ($\times 10^{-8}$)
h^-	0·85	3·4	2·7
h^-_α	0·75	3·0	3·2
h^-_β	0·83	3·3	3·0
h^-_γ	0·70	2·8	4·6
h^-_δ	0·96	3·8	4·2
h^-_ϵ	0·77	3·1	3·1
Average	0·81	3·2	3·5

of histidine supplied and, as a consequence, their presence in a culture was revealed by the development of heavy turbidity through adaptive overgrowth. Each experiment involved more than 100 cultures and the number of unadapted cultures was determined with the caution described in the footnote, p. 372. These unadapted cultures were those in which no mutation had occurred during growth. From this zero class the average number of mutations/culture could be calculated by the zero term of the Poisson formulation, e^{-m}, where m is the mean number of events. With a knowledge of m and number of bacteria-generations that had ensued, $N/\ln 2$ (the average total number of bacteria/culture, N, was determined by assaying at least three unadapted cultures), the mutation rate, a, could be calculated by the use of the equation defining mutation rate:

$$a=\frac{m}{N}\ln 2. \qquad (9)$$

As is shown in Table 3, the average mutation rate so determined is $3{\cdot}5\times 10^{-8}$/bacterium/generation. It can be seen that this is almost exactly 4 times the slope of the h^+ increase curves. Since the Poisson method uses only information about cultures without mutants and makes no assumption about how mutants appearing in the other cultures grow, mutation rates calculated by it are unaffected by segregation lag. The slope of the increase curve, on the other hand, is a function of the length of segregation lag. The comparison made in Table 3 shows a discrepancy that is not only inconsistent with the hypothesis of no segregation lag but is precisely that expected if segregation lag involves the separation of four mutable units and lasts two generations.

DISCUSSION

The evidence described in this paper, indicating the existence and the duration of a lag in the increase of mutant clones due to the segregation of nuclei, is rather indirect. Further, the models which were tested, being discontinuous and determinative, are at best only approximations. Other more realistic models can and have been constructed (Kendall, 1953; Armitage, 1953) but are difficult to test. There are also other conceivable differences between old and new mutants which might account for the data. Especially because data consistent with the hypothesis of a two generation segregation lag have been obtained for radiation-induced mutants by methods that directly measure the delay in the onset of growth of the mutant clone (Ryan *et al.* 1954), the hypothesis of a segregation delay of two generations has been accepted despite the reservations with which it must be held. This segregation lag has nothing to do with the long physiological delay observed in the division of new mutants after heavy doses of ultraviolet light (Ryan, 1954).

The operation of segregation lag has not usually been assumed in previously published methods for the calculation of mutation rate based upon numbers of mutants (Armitage, 1953). Where segregation lag is present these methods will not yield reliable figures, for segregation lag not only depresses the number of mutants but distorts their distribution. If, on the other hand, the curves that fit the data in Figs. 2 and 3 are taken to the level of one mutant on the ordinate and the total number of bacteria present at the time of the first mutation substituted in equation (9), rates of $3{\cdot}5\times 10^{-8}$ and $2{\cdot}3\times 10^{-8}$ are obtained. The average, $2{\cdot}9\times 10^{-8}$, is also not significantly different from the value $2{\cdot}7\times 10^{-8}$ obtained by the Poisson method and shown in Table 3.

The Poisson method would give an erroneous result if there were a physiological delay in the development of the phenotype, and some time were required for a bacterium, genotypically mutant, to become phenotypically mutant (Newcombe, 1948). If, for example, one generation were required for the expression of the phenotype, those mutations occurring during the last generation of growth would not give rise to phenotypically mutant bacteria. Since these mutations are always approximately equal in number to all that have occurred beforehand (see the models discussed above), the true rate of mutation would be underestimated by half. For reasons presented elsewhere (Ryan *et al.* 1954) it is believed that phenotypic lag does not operate in the system $h^- \rightarrow h^+$. It appears that the ability to synthesize histidine is developed in considerably less than a generation after the cell becomes genotypically h^+, and, because the h^+ character is expressed in a heterocaryotic cell in the presence of h^- genes, it is 'dominant'. This is the condition required for the action of segregation lag in the way found to be the case in the h^- to h^+ mutation.

If two generations of segregation lag actually take place, if the rate of mutation from h^- to h^+ has been accurately measured, and if periodic selection is responsible for the observed pseudo-equilibrium proportion of h^+ mutants (Atwood *et al.* 1951), it should be possible to calculate the dynamics of change in a mutating population of h^- bacteria in terms of experimentally determinable variables. The average time required for the beginning of overgrowth by fitter types of marked h^+ mutants added to serially transferred h^- cultures is about 200 generations. This, then, is the time at which the h^+ mutants of spontaneous origin (and their h^- parents) begin to decrease and, therefore, the time at which the pseudo-equilibrium is achieved. By the use of the formulation for two generations of segregation lag:

$$\frac{\text{Mutants}}{\text{Parents}} = \frac{ga}{4} + C_2, \tag{8}$$

an average mutation rate of 3×10^{-8} and the value C_2 calculated to be $4{\cdot}3 \times 10^{-8}$, the substitution of 200 generations should yield the pseudo-equilibrium proportion of mutants. The value calculated is *c.* $1{\cdot}5 \times 10^{-6}$ while that observed is *c.* $1{\cdot}3 \times 10^{-6}$. Thus the pseudo-equilibrium is quantitatively accounted for. The ability to describe accurately the course of change of h^+ mutants in an h^- population is additional evidence that the assumption of a segregation lag of two generations is valid.

Under different conditions and during different phases of the growth cycle the average number of nuclei/cell may differ (Robinow, 1945). If the nuclear bodies are, as they appear to be, the mutable units, then calculation of the mutation rate on a per bacterial cell basis would be misleading. Rather, to study environmental factors with only small effects on mutation rate it is necessary to calculate mutation rates on a per mutable unit basis. In the case of the mutation h^- to h^+ the spontaneous rate is *c.* 8×10^{-9} per mutable unit per generation during growth.

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Nuclear Segregation and the Growth of Clones of Bacterial Mutants induced by Ultraviolet Light

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SUMMARY: Histidine-independent (h^+) mutants induced in histidine-requiring (h^-) cultures by ultraviolet light have a delay in the onset of logarithmic increase that is about two generations longer than the delay shown by marked unirradiated h^+ bacteria present at the same time. This extra delay is interpreted as being due to the segregation of one from four nuclei which are present, on the average, in growing h^- organisms. The same assumption accounts for the extra delay observed in spontaneous h^+ mutants. These and other results are discussed in relation to the site of mutation and to the various types of delay that can retard the onset of growth of a mutant clone. It is concluded that in the mutation from h^- to h^+, the h^+ condition is dominant in the heterocaryon, that whatever phenotypic delay exists is short, and that cell division is not required to pass through it.

In the previous paper (Ryan & Wainwright, 1954) evidence was presented that led to the conclusion that growing *Escherichia coli* organisms have their genetic material represented at least 4 times, on the average, in each bacterium. In histidine-dependent (h^-) bacteria, the mutation leading to a histidine-independent condition (h^+) occurs independently among members of each set. Thus bacteria are produced which are initially heterocaryotic. Only after growth and division does the heterocaryon segregate its nuclei so that a homocaryotic mutant offspring is produced and only then does the clone of mutants increase by doubling. This delay is referred to as segregation lag and, in the system studied, lasted for a minimum of two generations. The evidence came from the overall rate of increase of mutants in growing populations of mutating parental bacteria and was therefore indirect. It seemed desirable to perform experiments with populations in which a large crop of new mutants had just been induced. The number present would not be expected to increase until they had become homocaryotic by the segregation of their non-mutant nuclei during growth. The length of the delay would be a function of the average number of nuclei per bacterium according to the relation:

$$\text{generations of segregation lag} = \frac{\log \text{no. of nuclei/bacterium}}{\log 2}. \qquad (1)$$

This possibility was approached directly by exposing h^- bacteria to ultraviolet light until more than 90% of the h^+ mutants present were induced mutants. The culture was then spread on the surface of agar devoid of histidine where only the h^+ mutants were able to grow and form colonies. From time to time the surfaces were re-spread in a way that separated all of the growing bacteria present. Only after segregation lag was over did the number of h^+ colonies increase. Since the irradiated culture had to be washed in order to remove histidine from the h^- parents and prevent their growth, and had to be plated from liquid on to an agar surface, a physiological lag was induced. To measure the length of the delay from these causes, cultures of h^+lac^- bacteria that could not ferment lactose were spread with the h^- bacteria at the beginning of the experiment. The difference between the delay in increase of numbers of new h^+ mutants and added marked h^+ bacteria was taken as a measure of the length of segregation lag because the added h^+lac^- mutants were already homocaryotic for h^+ nuclei. The results of these experiments were consistent with the existence of an average of four nuclei per growing bacterium. Furthermore, when similar procedures were applied to cultures containing spontaneous mutants, only a fraction of which were less than two generations old, the results led to the same conclusion.

MATERIALS AND METHODS

The strains and basic techniques used in these experiments have been described elsewhere (Ryan & Wainwright, 1954; Ryan, 1954). Ultraviolet light was used to induce new histidine-synthesizing (h^+) mutants because they were not found after treatment of h^- bacteria with such non-toxic mutagens as caffein, theophylline or $MnCl_2$ (the h^- locus is apparently stable in the presence of some mutagens; Demerec & Cahn, 1953). Appropriate inoculations of a histidineless strain (h^-) of *Escherichia coli* were made into 1 l. flasks containing 250 ml. lots of chemically defined salt medium with 0·05% (w/v) glucose and a concentration of histidine which yielded optimal growth. After overnight agitation at 37° the bacteria were in the logarithmic phase of growth and at a concentration of *c.* 2×10^8 organisms/ml. The cultures were washed once by centrifugation in 0·9% NaCl, resuspended in one-tenth of the original volume and distributed to Petri dishes in 10 ml. lots. In this condition they were illuminated while being rotated at a distance of 14 cm. from a Westinghouse 'Sterilamp'. The suspensions were then pooled, washed once in saline and resuspended so as to make a total concentration of one-hundred-fold. All operations after the irradiation were performed in the yellow light of a G.E. 'Bugaway' lamp to prevent photo-reactivation.

The cultures were diluted when necessary and spread by the use of a sterile glass rod and a rotating stand on to the surfaces of agar plates containing or not containing histidine. After low doses of ultraviolet light (<40 sec.) the resulting colonies were easily countable after 2 days incubation at 37°; after higher doses of irradiation it was necessary to wait 3 days before all of the induced h^+ mutant colonies developed. This is because the h^+ mutants induced by high doses of ultraviolet light appear in a fraction of the population that has a very long induced delay in the onset of division (Ryan, 1954). Fig. 1 shows the results obtained with a variety of doses of ultraviolet light. As the survival drops to 10^{-5} the frequency of induced h^+ mutants among the survivors rises to between 10^{-4} and 10^{-3}. The mutant increase curve is extremely steep after an initial lag and this may account, in part, for the failure of Lieb (1951) to induce a considerable number of h^+ mutants in this strain with doses of ultraviolet light which left as many as 10^{-3} survivors. It is interesting to note that the killing curve of these h^- bacteria is exponential down to *c.* 10^{-4}

or 10^{-5} under the conditions used. Since the organisms in growing cultures contain an average of more than one nuclear body, it may be concluded that nuclear inactivation is not the main cause of bacterial inactivation. An h^- survivor of 120 sec. of irradiation, when isolated, grown and re-irradiated, gave survival and induced mutant curves indistinguishable from those of its parent. It does not seem probable, therefore, that the break in the survival curve shown in Fig. 1 was due to a small fraction of the population of bacteria being genetically resistant to ultraviolet light.

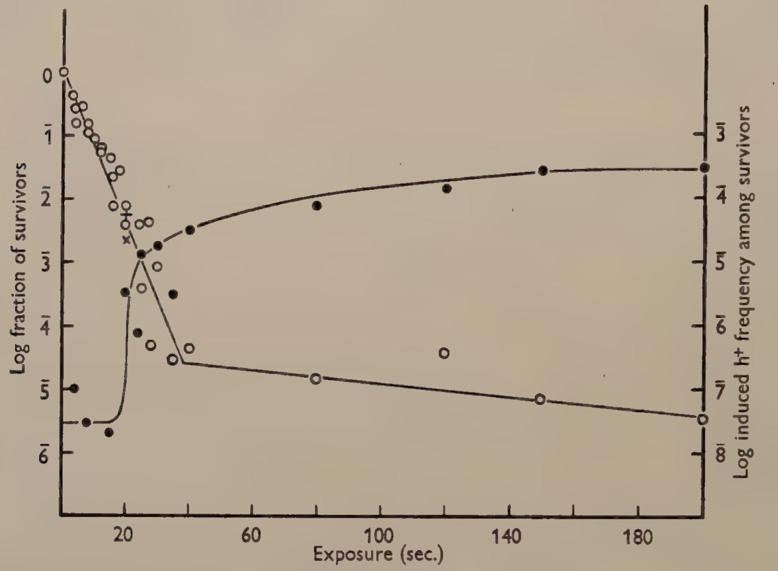

Fig. 1. Survival of, and induced h^+ mutant frequency among, h^- bacteria removed from the condition of logarithmic growth and exposed to ultraviolet light for various periods of time. The left ordinate measures the $\log_{10}$ of the fraction of survivors (open circles), the ordinate on the right, the $\log_{10}$ of the frequency of induced mutants among the survivors (black circles); and the abscissa, the length of exposure to ultraviolet light in seconds. The data for 20 sec. are the averages of 15 experiments and were therefore weighted in drawing the curves (the black circle that lies out of relation to its mates is based upon a single determination). The average frequency of spontaneous h^+ mutants was $3{\cdot}4 \times 10^{-7}$. The × is for the survival of an h^+ mutant previously secured from the h^-; the + is for an h^+lac^- strain derived from the h^- in two steps.

For the experiments to be reported in this paper a dose of 20 sec., yielding an average survival of 4×10^{-3}, was chosen. This dose seemed suitable because: (i) it usually resulted in more than 90 % of the h^+ mutants being induced and new; (ii) it did not produce the extremely long extra physiological delay in the onset of growth of the induced h^+ mutants found after high doses of ultraviolet light (Ryan, 1954); (iii) it did not appear to inactivate the washed bacteria in such a way as to enable them to decompose and yield appreciable histidine to the medium. Fig. 2 shows the result of spreading 0·2 ml. of an irradiated culture on a series of agar plates devoid of histidine and, at intervals thereafter, washing the suspensions from the surfaces by scrubbing in two successive lots of 5 ml. of added sterile saline with the aid of a sterile glass rod and a turntable. The two lots were pooled, washed and plated on agar with and without histidine for differential assay of viable h+ and h− bacteria. The washing-off procedure did not result in a detectable loss of bacteria. In three experiments designed to test the efficiency of this procedure an average of 90 % of the h− and 114 % of the h+ bacteria estimated to have been on the plates were recovered by washing off.

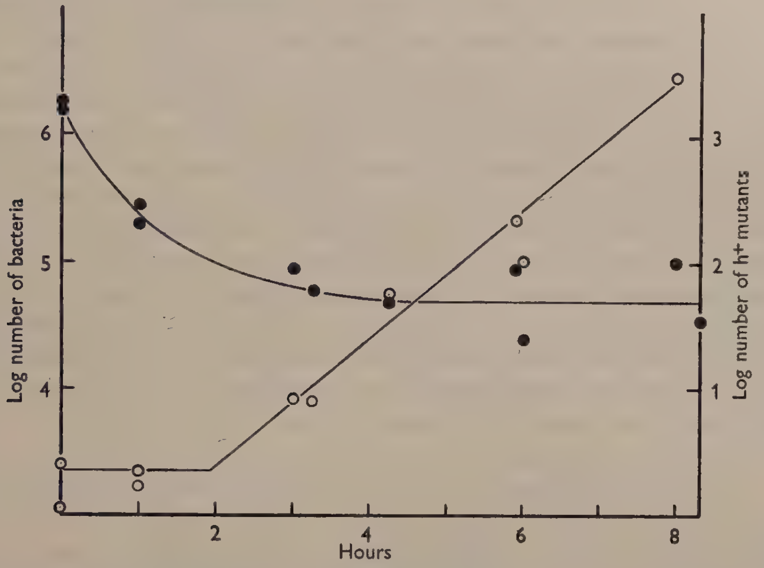

Fig. 2. The behaviour of irradiated h− bacteria (black circles) and of the h+ mutants (open circles) induced among them after being spread on agar devoid of histidine. The left ordinate measures the $\log_{10}$ of the number of viable h− bacteria/plate; the ordinate on the right, the $\log_{10}$ of the number of viable h+ bacteria/plate; and the abscissa, the time in hours at which they were washed from the plates for assay. The data come from 2 experiments where the average survival was $6 \cdot 3 \times 10^{-4}$ but where only 56 % of the mutants present were induced. The generation time for the h+ bacteria during logarithmic growth was 35 min.

It can be seen from Fig. 2 that the surviving h+ bacteria grew normally after a short lag (a generation time of about 35 min. instead of 55 min. was usually found during logarithmic growth of cultures surviving an irradiation of 20 sec.; this is probably due to the release of some stimulating materials by the inactivated organisms). The surviving h− bacteria did not find histidine on the plates with which to grow and divide but, as a matter of fact, underwent considerable death upon incubation.* After more than 10 hr. they appeared to autolyse and release a little histidine; after 22 hr. the numbers of viable h− bacteria on the plates in the experiments shown in Fig. 2 had increased from

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* Death of irradiated bacteria of a similar sort was discovered by Lieb (1951). She found that a large fraction of irradiated bacteria quickly died when suspended in liquid medium but survived when agar was present. The phenomenon was called post-irradiation death and has been confirmed by us. Furthermore, although it does not occur on agar containing histidine it does occur on agar devoid of histidine (Fig. 2) provided that the bacteria have not been so heavily irradiated that histidine is released by the autolysis of inactivated bacteria (Ryan, 1954).

$1{\cdot}9 \times 10^6$ to $7{\cdot}4 \times 10^6$. But this increase occurs far too late to have any influence on the initial increase in number of h^+ bacteria.

In most of the experiments to be reported the bacteria were not washed from the plates but were re-spread at some later time in 0·2 ml. added sterile saline. Since there was no histidine on the plates, only the h^+ mutants were able to form colonies: it was in the course of this growth that the clones were separated by the re-spreading procedure. Evidence that the sister bacteria were efficiently separated comes from the observation of the normal logarithmic growth curves resulting; for example, unirradiated h^+ bacteria were observed to have a generation time of 51 min. by this method as compared to 50–55 min. by other methods but under similar conditions. Furthermore, the number of h^+ mutant bacteria present on the plates at any time, as determined by the spreading or the washing-off techniques, was the same.

In order to interpret the length of the delay of the new h^+lac^- or h^+lac^+ mutants, h^+ bacteria from established stocks and carrying either a lac^+ (lactose-fermenting) or a lac^- (non-lactose-fermenting) marker were added in appropriate amounts to the irradiated h^- cultures just before the latter were first spread. The combinations used are described in Table 1. All of the strains used had, in tenfold concentrations and when taken as they were from the growing condition, the same sensitivity to ultraviolet light (Fig. 1).

Table 1. *Variations in the mixtures introduced on the surfaces of plates devoid of histidine and respread*

Mixture	Strains	Volume grown in (ml.)	Irradiated	Final concentration	Results described in
A	h^-lac^+ h^+lac^-	250	Yes Yes	100×	Fig. 3
B	h^-lac^+ h^+lac^-	250	Yes No	100×	Fig. 4
C	h^+lac^+ h^+lac^-	100	Yes No	10×	Fig. 5
D	h^+lac^+ h^+lac^-	100	No Yes	10×	Text
E	h^-lac^+ h^+lac^-	100	No No	1×	Fig. 6

Experiments designed to test for the possibility of suppression of the growth of h^+ mutants on the crowded plate surfaces were performed. First, suspensions of h^- bacteria were irradiated for 20 sec., concentrated as usual and then spread on plates devoid of histidine in dilutions ranging around that used routinely in the re-spreading experiments. The numbers of h^+ colonies found were appropriate for the dilution. Secondly, in nine experiments where an estimated average of 14 marked h^+ bacteria had been added to plates containing irradiated h^- or h^+ organisms, 19 marked h^+ colonies were recovered on the average. Hence with the method used the results were not influenced by suppression of h^+ colonies nor by mutations occurring on the plates.

Furthermore, in ten experiments where an estimated average of 9 marked h^+ bacteria were added to plates devoid of histidine but containing the unirradiated h^- suspensions used in the studies of the delay in the growth of spontaneous h^+ mutants, 11 marked h^+ colonies were recovered on the average; and in four experiments where an estimated average of 12 marked survivors of a suspension of h^+ bacteria irradiated for 120 sec. were added to plates containing h^- organisms irradiated to the same extent, an average of 10 marked h^+ colonies was found. Hence, the conditions used in the experiments reported here and elsewhere (Ryan, 1954) did not suppress h^+ colonies.

In all spreading experiments the increase in numbers of new and added h^+ mutants was followed by the use of two series of plates all devoid of histidine. On one series 0·1 ml. of only the irradiated h^- (or h^+) bacteria was spread; on the other, the same bacteria and the added mutants were spread together. On the latter plates discrimination between the marked and unmarked (lac^- and lac^+) bacteria was achieved with eosin methylene-blue agar by the replication technique of Lederberg & Lederberg (1952). The numbers of the new (or, in some cases, irradiated) h^+ bacteria were taken as the averages of the colony counts on the first series of plates and the counts of the proper lac type on the replicate plates. The numbers of the added component were taken directly from the replicate plates; when these were too crowded for accurate reading, the numbers were taken as the difference between the counts on plates of the first and second series.

Cytologica lstudies were made on preparations of growing h^- bacteria taken from defined medium + histidine. The procedures adopted were essentially those of Robinow (1945) and involved Giemsa stain.

RESULTS

Fig. 3 shows the growth curves for h^+ mutants arising in irradiated h^-lac^+ cultures and of h^+lac^- mutants that were irradiated to the same survival and mixed with them. The lag period for both mutant types was the same and lasted for a little more than 2 hr. or a time equivalent to about 4 generations. Since both of the h^+ mutants were irradiated it is very likely that only one surviving nucleus was present/surviving bacterium.* The segregation of viable from inactive h^+ nuclei in the h^+lac^- strain and of viable h^+ from inactive h^- nuclei in the new mutants is probably the cause of the equal delays in growth. Similarly, in two experiments, where irradiated h^- cultures were spread on plates containing histidine and then washed off for a differential assay of h^+ mutants and h^- parents, no significant difference could be observed in the times of onset of growth. The proper control thus appeared to be added h$^+$lac$^-$ mutants which had not been irradiated.

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* These h^+ bacteria had been exposed to a dose of ultraviolet light on the exponential part of the killing curve (Fig. 1). As mentioned earlier, this curve is not a function of the cumulative inactivation of the nuclei present. Nonetheless, when more than 99 % of the bacteria have been killed it seems probable that three of the four nuclei may have been inactivated among the survivors. The relation between nuclear inactivation and cell killing is clearly complex; there is not even reason to believe it is the same in the spores of *Neurospora* (Atwood, unpublished) where there are several nuclei in a common cytoplasm, and in growing *Escherichia coli*, where cell walls eventually separate the nuclei originally present.

Such an experiment is shown in Fig. 4 which describes the growth of h$^+$ mutants produced by the irradiation of an h$^-$lac$^+$ culture and of unirradiated h$^+$lac$^-$ bacteria which were spread on the same plates at the beginning of the experiment. The difference in the times of onset of growth is estimated as equal to 1·8 generations, or 1·9 generations if the extra delays in the three individual component experiments are separately determined and averaged. These estimated delays are not significantly different from that of 2 generations expected for the segregation of four nuclei.

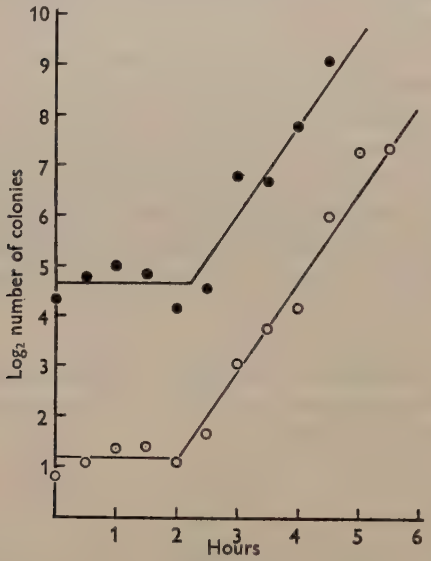

Fig. 3

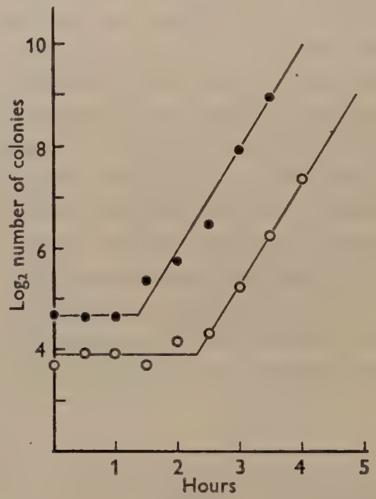

Fig. 4

Fig. 3. The growth of h$^+$ mutants (open circles) found among irradiated h$^-$lac$^+$ bacteria spread on the surfaces of agar devoid of histidine and of irradiated h$^+$lac$^-$ bacteria (black circles) that had been added to them. The ordinate measures the log$_2$ of the number of colonies/plate; the abscissa, the time (hr.) at which the plates were respread. The data are the averages of 2 experiments where the average survival was $1{\cdot}3 \times 10^{-3}$ and where 98 % of the mutants present were induced. The generation time during logarithmic growth was 34 min.

Fig. 4. The growth of h$^+$ mutants (open circles) found among irradiated h$^-$lac$^+$ bacteria spread on the surfaces of agar devoid of histidine and of unirradiated h$^+$lac$^-$ (black circles) that had been added to them. The ordinate measures the log$_2$ of the number of colonies/plate; the abscissa, the time (hr.) at which the plates were respread. The data are the averages of 3 experiments where the average survival was $1{\cdot}9 \times 10^{-2}$ and where 99 % of the mutants present were induced. The generation time during logarithmic growth was 30 min.

When unirradiated h$^+$lac$^-$ bacteria were added to irradiated h$^+$lac$^+$ cultures the results shown in Fig. 5 were obtained. The irradiated mutants had an extra lag of 1·6 generations as calculated from the average curve or of 1·8 generations when the extra lags in the individual component experiments were determined and averaged. In one experiment involving the mixture of irradiated h^+lac^- and unirradiated h^+lac^+ bacteria, the lag of the irradiated bacteria was 1·3 generations longer than that of the unirradiated. In view of the errors involved in this sort of experiment and the observed variability (1·3–2·3 generations) it is concluded that the lac marker is neutral and that the extra delay in the onset of growth of new mutants is close to 2 generations.

When the length of exposure to ultraviolet light is increased to 120 sec. so that survival is reduced to *c.* 10^{-5}, an extremely long delay lasting sometimes for more than 30 generations is induced in new h^+ mutants (Ryan, 1954). The 2-generation delay following a 20 sec. exposure to ultraviolet light could conceivably be of this physiological sort. The physiological delay after high doses of ultraviolet light affected the mutant, not the parent, and was extremely variable, while, on the other hand, the 2-generation extra delay is consistent and unspecific. Furthermore, the eventual onset of growth is of an abruptness not expected if the delay had a physiological cause or were due to induced irregularities in the onset of division of individual members of the population (Newcombe & Scott, 1949). Perhaps the strongest argument for nuclear segregation as the cause of the 2-generation delay is that the assumption of an average of four nuclei accounts not only for it, but also for delays in growth of spontaneous mutants (Ryan & Wainwright, 1954). This is also true when spontaneous mutants are studied by the same methods that were used for the induced mutants.

Because of the growth of earlier mutants, the proportion of spontaneous mutants less than 2 generations old continually decreases during the growth of a culture. By the use of the model for the increase in numbers of spontaneous mutants on the assumption of 2 generations of segregation lag (Ryan & Wainwright, 1954; see also Table 2 below), the proportion in the mutant population of mutants which have existed for any given number of generations can be calculated for any population size. In the experiments to be reported, 100 ml. cultures of h^- bacteria, each containing a total of *c.* $3{\cdot}7 \times 10^3$ h^+ mutants, were used. Among this number of mutants it is calculated that *c.* 27 % would be less than one generation old and would not increase the population of mutants until two generations of growth had ensued; 13·5 % would be one generation old and would 'lag' for only one generation; the remainder would be out of lag and would grow as rapidly as their parents. Such a mixed population of mutants would be expected to increase less rapidly than a pure culture of mutants homocaryotic for the h^+ gene, and the extra delay is calculated as *c.* 0·5 generations.

When such mixtures were made, using h^-lac^+ cultures and added h^+lac^- bacteria and were respread on the surfaces of plates devoid of histidine, the results shown in Fig. 6 were found. The added mutants increased abruptly after a delay of a little more than 1 hr. The mutant population, including mutants still in segregation lag, increased gradually with an extra delay of *c.* 0·7 generations as calculated from the composite curve or 0·6 generations when the estimate is the average extra delay found in the individual component experiments. These values were not significantly different by *t* test

(*P* is between 0·5 and 0·6) from the 0·5 expected. Thus, once again, the data were consistent with the assumption of an average of four nuclei per bacterium and consequent segregation lag.

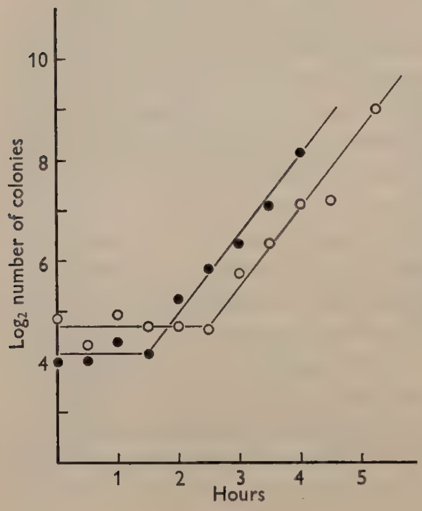

Fig. 5

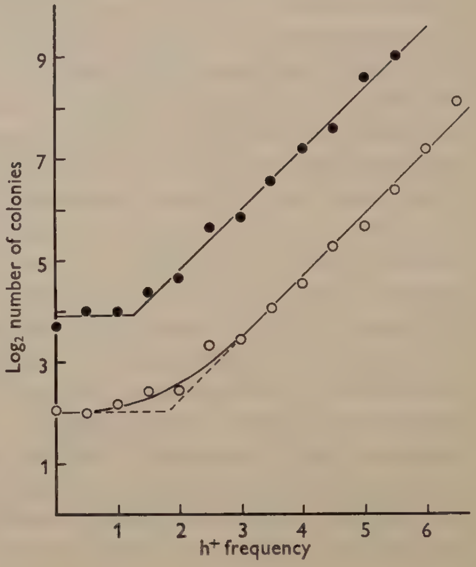

Fig. 6

Fig. 5. The growth of irradiated h^+lac^+ bacteria (open circles) spread on the surfaces of agar devoid of histidine to which unirradiated h^+lac^- bacteria (black circles) had been added. The ordinate measures the $\log_2$ of the number of colonies/plate; the abscissa, the time (hr.) at which the plates were respread. The data are the averages of 3 experiments where the average survival was $2{\cdot}2 \times 10^{-3}$. The generation time during logarithmic growth was 38 min.

Fig. 6. The growth of h^+ mutants (open circles) of spontaneous origin among unirradiated h^-lac^+ bacteria spread on the surfaces of agar devoid of histidine and of unirradiated h^+lac^- bacteria (black circles) that had been added to them. The ordinate measures the $\log_2$ of the number of colonies/plate; the abscissa, the time (hr.) at which the plates were respread. The data are the averages of 8 experiments where the average spontaneous h^+ mutant frequency was $4{\cdot}0 \times 10^{-7}$ (median $= 1{\cdot}7 \times 10^{-7}$). The generation time during logarithmic growth was 51 min.

DISCUSSION

The word 'nucleus' has been used throughout this paper as if it were synonymous with 'mutable unit'. The identification of nuclei as the sites of mutation must rest eventually on cytological studies. During the logarithmic phase of growth h^- bacteria seem to have mostly two nuclear bodies/cell. However, there is also a fair-sized class of cells containing what appear to be four nuclear bodies; in addition, there are rare cells containing one, three or more than four bodies and, in our preparations, quite a few bacteria in which the number of nuclear bodies cannot be counted accurately. In view of the difficulty in resolving nuclear bodies in bacteria which have been grown in chemically defined media we cannot be sure that what appear to be single nuclear bodies are not double. It would seem quite possible that newly divided bacteria have two visible nuclear bodies, in each of which the genetic material has already duplicated (Robinow, 1945, p. 358). During the growth and elongation of the cell the two bodies separate into four, each of which now has only one set of genetic material. At the time of cell division the genetic material divides and the number of nuclear bodies is reduced to two. The morphological and spatial relationships to be seen among the dividing nuclear bodies would suggest that their separation is regular. If it were irregular the lag in the onset of mutant increase would simply be interrupted giving a gradual average rise at the beginning of the growth curve. The abrupt rise observed speaks for the regularity of nuclear separation. The mutable units, like nuclei, seem to be independent of one another in mutation, to segregate in a regular fashion and to be represented four times on the average in each bacterium.

There are several types of delay that can differentially retard the onset of increase of a mutant population. The more obvious of these can be classified as: (1) delay in cell division; (2) delay in the mutational event; (3) segregation delay; (4) phenotypic delay.

All bacteria exhibit a delay in the onset of division when they are taken from a phase of logarithmic growth into some other and then returned to the original growth-supporting medium. Cultures in the stationary phase or logarithmically growing bacteria that have been washed, show such a physiological delay, but it is usually the same for mutant and parent. It may be longer for the mutant under some conditions such as those induced by a very strong dose of ultraviolet light. In this case the new mutants seem to appear in that fraction of the population which the irradiation has specifically delayed (Ryan, 1954). But doses of this sort, yielding survivals of *c.* 10^{-5}, are much greater than those used in the experiments reported above which resulted in survivals of *c.* 10^{-3} and which did not specifically delay the new mutants (Fig. 3). A delay in the mutational event for some time after the application of the inducing stimulus seems to be a real possibility in some instances (Witkin, 1951; Kaplan, 1952; Newcombe, 1953). Such a delay cannot account for the results reported in this paper nor do these data shed any light on the question of mutation delay.

Segregation lag and phenotypic delay are clearly distinguishable in principle and in practice. The former has consequences of the sort described in this paper while the latter, being a delay in the biochemical development of the phenotype, demands that the mutant fail to appear as such until the phenotype is expressed. Thus bacteria possessing an h^+ genotype would be counted as h^- as long as development of the histidine-synthesizing system, which makes the bacterium phenotypically h^+, is not completed. As a consequence the frequency of mutants, and in Poisson experiments the fraction of cultures with one or more mutants, would be underestimated. Because when the number of mutants is large the fraction of recent enough origin to be still in phenotypic delay may be small, methods of measuring mutation rate that depend upon the number of phenotypically expressed mutants present will not be as seriously affected by phenotypic delay as methods depending upon the fraction of cultures without mutants. In the latter case, if there were, for example, one generation of phenotypic delay, the fraction of cultures without mutants would be that determined by all mutations except those which occurred during the last generation of growth. Since at each generation a number of mutations occur which is, on the average, approximately equal to all preceding mutations (Table 2), the true number of mutations will be underestimated by half. The total number of bacteria, on the other hand, is correctly determined. Consequently the mutation rate, a, will be underestimated by half for every generation of phenotypic lag when the formula is used that defines mutation rate

Table 2. *Expected average increase in the number of mutants on the assumptions of random mutation, no selection and a segregation lag of 2 generations*

Generation	Parents	Mutants New Old	Total no. of mutants
1	n	$1 \; \overbrace{\quad}$	1
		↘	
2	$2n$	$2+1$	3
		↘ ↘	
3	$4n$	$4+2+1$	7
		↘ ↘ ↘	
4	$8n$	$8+4+2+2$	16
—	—	— — — —	—

$$a=\frac{m}{N}\ln 2, \tag{2}$$

where $N/\ln 2$ is the total number of bacteria-generations that have existed during the growth of the culture and m is the average number of mutations, based upon the proportion of cultures without mutants and on the zero term of the Poisson formulation, $P_0=e^{-m}$.

In the first specific study of phenotypic lag following bacterial mutation, Newcombe (1948) explained on that basis the low rate of mutation of *Escherichia coli* to resistance to bacteriophage T_1 when determined by the Poisson method as compared with methods based upon the numbers of mutants present in different cultures. But the latter methods assume that the distribution of the numbers of mutants is in accord with the assumptions of random mutation, equal growth of parent and mutant and no segregation lag. If these assumptions are correct, the distribution of T_1 resistant mutants should be that calculated by Lea & Coulson (1949); but it is not (Ryan, 1952). Consequently, mutation rates calculated on the basis of numbers of mutants are unreliable and discrepancies between them and rates calculated from the fraction of cultures without mutants do not demonstrate the operation of phenotypic lag. Furthermore, working with mutations to streptomycin resistance, which is a recessive character in *E. coli* (Lederberg, 1951) and one whose distribution among cultures would not be expected to be, and was not observed to be, affected by segregation lag, Newcombe & Hawirko (1949) found no discrepancy between mutation rates calculated by the two types of methods. Hence, mutation rate comparisons have not yet yielded evidence of phenotypic lag.

Whenever phenotypic lag lasts for more than a generation no new mutants can appear until growth has ensued. Demerec (1946) has interpreted in terms of phenotypic lag the failure to find induced phage-resistant mutants until after a period of growth had intervened. This and the delayed appearance of mutants in general may, however, have other interpretations (Ryan, 1954). The contention of Lieb (1951) that ultraviolet-induced h^+ mutants do not express themselves without a period of intermediate cultivation has not been confirmed (Kaplan, 1950; Ryan, 1954). Working with other mutations to prototrophy, Davis (1950) found that after treatment with ultraviolet light the number of prototrophic mutants was increased when a small amount of the growth factor required by the parents was added to the cultures. The role of the added growth factor is not clear in this case and, as a consequence, neither is the role of phenotypic lag. There must, of course, be some time required for phenotypic expression but it is a question whether this time is as great as one or more generations.

Segregation lag and phenotypic delay could very well operate simultaneously. If the mutation involved were 'recessive' in a heterocaryon, phenotypic delay would begin after the mutant became homocaryotic for mutant nuclei. Thus, the mutant could not express itself without previous growth. If, on the other hand, the mutation were 'dominant' in a heterocaryon, phenotypic delay would begin at the moment of mutation. If the development of the phenotype required, for example, one generation and there were two generations of segregation lag, growth would not take place in the absence of the growth factor required by the parent; hence, the mutant would not be detected. If the growth factor were supplied, however, development of the phenotype could be achieved. But during this period, until the end of which the mutant would be cryptic, one generation of segregation lag would have occurred. From the time of appearance of the mutant only one generation of segregation delay would be observed. Thus a generation of phenotypic delay would conceal one generation of segregation and the mutation rate, determined from the frequency of cultures without mutants would be halved by each generation of phenotypic delay. These points can be seen perhaps more clearly if one imagines that all of the new mutants listed in the appropriate column in Table 2 are in phenotypic lag and hence are cryptic.

Thus, induced h^+ mutants, on any assumption of phenotypic delay, would not be expected to be detectable without growth, and yet they are (Ryan, 1954). This fact is most clearly shown in Fig. 2 of this paper. After irradiation, bacteria of h^- phenotype not only fail to grow, but die on agar devoid of histidine; yet new h^+ mutants express themselves and grow normally—they are zero point mutants. Therefore, no division is needed for the expression of the h^+ phenotype. It can be further concluded that the h^+ mutation is dominant in heterocaryons on the following argument. The development of the h^+ phenotype is required for growth on agar devoid of histidine. On such agar induced h^+ mutants do appear and multiply while bacteria of h^- phenotype do not. Therefore, the h^+ phenotype must have been imposed without previous division. Since there is a delay in the initiation of the mutant clone due to the segregation of nuclei, the newly mutated h^+ gene must have influenced the h^+ phenotype even in the presence of its h^- sisters which were later segregated. Therefore, it was dominant in a heterocaryon.

It might be supposed that, in the heterocaryons formed upon mutation, the h^+ gene is recessive but that the three sister h^- nuclei were inactivated by irradiation (and also, perhaps, by the process leading to the death of h^- bacteria observed in Fig. 2). Thus, the cells would be effectively uninucleate and hence homocaryotic for the h^+ nuclei. The observed segregation delay would involve the separation of the active h^+ nucleus from its inactivated h^- sisters. But this hypothesis of h^+ recessivity would, further, have to assume that along with inactivation of h^- nuclei there was inactivation of all parts of the suppressive h^- phenotype. Furthermore, since delay in the onset of mutant increase does not take place when a mutation is recessive (assume in Table 2 that all new mutants are cryptic for two generations), this hypothesis will not account for the lag of spontaneous h^+ mutants occurring among unirradiated h^- bacteria which neither die nor grow when spread on agar devoid of histidine.

This latter fact was attested by six experiments in which an average of $5{\cdot}4 \times 10^4$ washed unirradiated h^- bacteria were spread on agar plates devoid of histidine and washed off again at various times up to 24 hr. There was a slight increase that ceased at a level of $7{\cdot}2 \times 10^4$ bacteria/plate. Thus, the agar could support the division of only *c.* 2×10^4 among the *c.* 2×10^7 h^- bacteria spread on the plates used in the spontaneous h^+ experiments. The chance that a heterocaryon, still phenotypically h^-, could divide is therefore only 10^{-3} at a maximum. Since the frequency of h^+ mutants recovered in medium with histidine (Ryan, 1954) is the same as that obtained in its absence, the very slight growth in the absence of added histidine could not mask a possible recessive nature of the h^+ gene.

A final argument regarding the role of phenotypic lag in the $h^- \rightarrow h^+$ system can be made as follows. From data on the spontaneous mutation it has been established that the length of segregation lag minus phenotypic lag is *c.* 2 generations (Ryan & Wainwright, 1954). Therefore, segregation lag has a minimum length of *c.* 2 generations. If the h^- mutable units respond to ultraviolet light independently, then the delay between irradiation and the onset of exponential growth of the induced h^+ mutant clone gives the delay due to segregation lag plus phenotypic lag. Hence, it established the maximum length of segregation lag also as *c.* 2 generations. By difference, phenotypic lag must be short, and lasts less than 1 generation.

The simplest conception, consistent with all available data, portrays the h^- bacterium as possessing, on the average, four mutable h^- genes. Mutation to the h^+ condition is spatially random and results in the formation of a heterocaryon. In this condition the newly mutated h^+ gene is able to evoke the development of the h^+ phenotype in a short period of time and without growth. Once the h^+ phenotype is developed growth occurs even in the absence of histidine. During this growth the h^- genes segregate regularly and after two divisions a cell homocaryotic for h^+ nuclei is formed. Only then does the h^+ mutant clone increase normally by doubling.

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CLARK, J. B. & WEBB, R. B. (1954). *J. gen. Microbiol.* **11**, 394–397.

Mitosis-like Figures in *Corynebacterium pseudodiphtheriticum*

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SUMMARY: By combining nucleus and cell-wall studies on the same bacterial cells, it was demonstrated that many mitosis-like configurations are actually divided by cross-septa. The results do not signify the absence of classical mitotic figures, but they do show that a nuclear study by itself is not sufficient to establish the existence of such configurations in the bacterial cell.

It is now generally agreed that bacteria contain a discrete body that resembles the nucleus of the cells of higher forms of life in both chemical composition and general activity. The division of this nuclear body is orderly since there is a uniform distribution of genetic determinants to the daughter cells. However, whether this division occurs in bacteria by a mitotic mechanism similar to that found in other cells has not yet been proven. DeLamater (1951, 1952*a–c*), DeLamater & Mudd (1951), DeLamater & Hunter (1951), and DeLamater & Woodburn (1952) offered evidence as to the existence of true mitosis in several bacterial species. This work has been criticized by Bisset (1950, 1952 *a*, *b*, 1953 *a*, *b*, 1954) as a misinterpretation of cytological events. It has been shown that a study of nuclear activity in bacteria without consideration of events of cytokinesis may lead to spurious interpretations (Chance, 1953*a*; Webb & Clark, 1954; Webb, Clark & Chance, 1954).

In the course of several cytological investigations on bacteria, nuclear configurations were sometimes observed which were strongly suggestive of various stages of a mitotic division. These mitosis-like figures were observed most frequently in *Corynebacterium pseudodiphtheriticum*. Since septation can easily be the basis of artefacts when a nuclear strain is used alone on bacterial cells (Bisset, 1954), a cell-wall study was used in conjunction with the study of nuclear figures.

METHODS

Nuclear studies were made by using a modification of the crystal violet stain (Chance, 1952). In this procedure a 0·5 % mercuric chloride solution was used for 20 sec. as a mordant. Selected fields for study were recorded by a vernier mechanical stage and photographs made on Kodak panatomic-X film with a Gamma photomicrographic camera. The oil was removed from the slide with toluene and the slide was then decolorized by soaking overnight in 50 % (v/v) ethanol in water before restaining to show cell walls. The cell-wall stain was a modification of the tannic acid-violet method (Webb, 1954). The microscopic fields studied for nuclear activity were again located and photographed. This gave both a nuclear and cell-wall study of the same cells. A similar procedure was used by Robinow (1945).

RESULTS AND DISCUSSION

A study of nuclear appearance and activity, *per se*, is not sufficient to prove the existence of true mitosis in bacteria. The minute details of the nuclear configurations are too near the limits of the resolution of the microscope to allow critical study of them alone. Also, small staining irregularities or artefacts could be interpreted with false significance. Bisset (1950) and other workers have pointed out the complex cellularity of bacteria, and have referred to them as multicellular organisms. This complex cellularity has been found in many bacteria in that the normally recognized morphological unit may not be a single cell, but instead may be divided by cross-septa. The presence of these septa is not seen by normal nuclear studies, and what appears to be, for example, the chromosomal masses of an anaphase may really be two nuclear masses separated by mature cross-septa.

As shown in Pl. 1, figs. 1–19, many nuclear configurations which could easily be interpreted as various mitotic figures are actually nuclear bodies in separate cells of the 'multicellular' organism.

Chance (1953*a*, *b*) has demonstrated the formation of cell plates in several genera of bacteria, and the cell-plate configuration may in many cases appear similar to a metaphase. In larger cells in which cell plates are formed, it is obvious that this formation could not take place before the anaphase and in most cases it has been found to occur during the telophase. The plate formation is the initiation of septum formation, and it has been found that the actual septum is not formed in bacteria until late in the development of the plate (unpublished data). Therefore, it can only be concluded that these mitosis-like configurations do not conform to the classical mitotic figures.

Similar configurations have been observed in other micro-organisms, but much less frequently than in *Corynebacterium pseudodiphtheriticum*. Since this project was intended primarily to explore the possibility of cross-septation leading to false interpretations, the study was concentrated on this one organism. It must be considered that the exact staining mechanism of the crystal violet nuclear stain is unknown. Intensive cytological studies have shown this procedure to be specific for bodies that display nuclear activity. However, preliminary work in our laboratory has indicated that the specificity of the stain is for protein components of the nucleus rather than for deoxyribonucleic acid. Thus this stain procedure is not directly comparable with the techniques routinely used in other laboratories. The general conclusions obtained by use of the crystal violet stain, however, should apply to bacterial cytology in general.

These results do not signify that classical mitotic figures do not occur in bacteria, but they do show that a nuclear study by itself is insufficient to establish their existence. It is possible that mitosis in bacteria occurs by some different, as yet unrecognized pathway. Perhaps in bacterial mitosis the timing of events of cytokinesis in relation to karyokinesis is different from that in other cells, and the cross-septum forms early in nuclear division. Such matters are still open to speculation on the basis of present information.

However, it is clear that there is little justification for claiming the existence of a true mitotic cycle in bacteria on the sole basis that some cells are seen to contain a nuclear mass which resembles a mitotic configuration.

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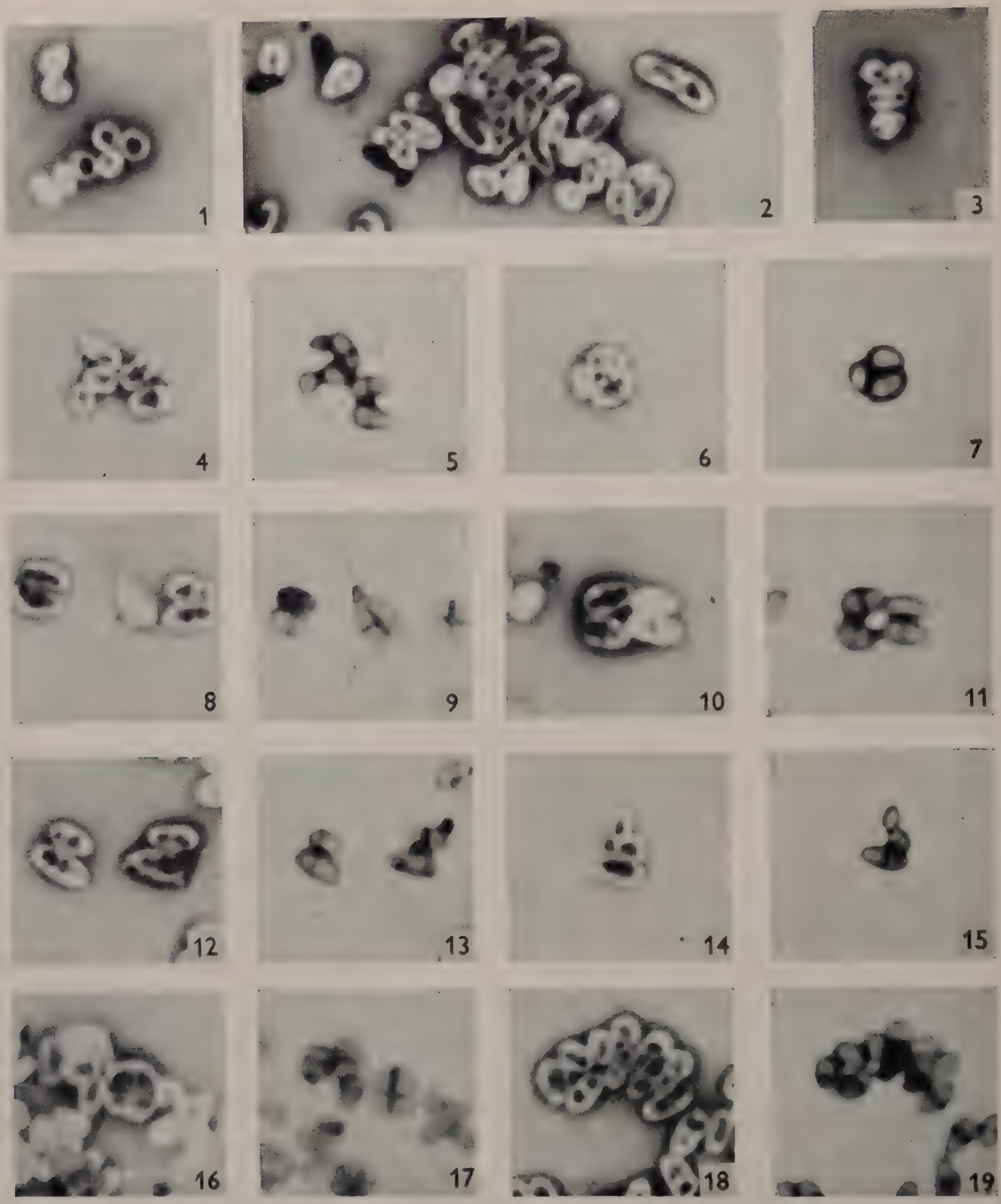
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J. B. Clark & R. B. Webb—Mitosis-like figures in bacteria

EXPLANATION OF PLATE

Mitosis-like figures in *Corynebacterium pseudodiphtheriticum* (All photographs × 3200).

Figs. 1–3. Variation in nuclear appearance in *Corynebacterium pseudodiphtheriticum.*
Figs. 4 and 6. Cell-plate formation.
Figs. 5 and 7. Cell-wall stains of cells shown in Figs. 4 and 6.
Fig. 8. Anaphase and metaphase configurations.
Fig. 9. Cell wall stain of organisms shown in Fig. 8.
Fig. 10. Metaphase configuration.
Fig. 11. Cell wall stain of organisms shown in Fig. 10.
Figs. 12 and 14. Anaphase configurations.
Figs. 13 and 15. Cell wall stain of cells shown in Figs. 12 and 14.
Figs. 16 and 18. Late anaphase or early telophase configurations.
Figs. 17 and 19. Cell wall stain of cells shown in Figs. 16 and 18.

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WARE, G. C. (1954). *J. gen. Microbiol.* **11**, 398–400.

The Effect of Incubation Temperature on the Growth Requirements of *Proteus vulgaris* and *Salmonella typhi*

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SUMMARY: *Proteus vulgaris* and *Salmonella typhi* require for growth at temperatures above normal complex nitrogenous substances additional to the media suitable for growth at normal temperature.

It was shown (Ware, 1951) that several strains of *Escherichia coli* and *Aerobacter aerogenes* which were able to grow at 37° in a defined medium were unable to grow in the same medium at higher temperatures. The addition of glutamic acid and nicotinamide to the medium supported growth up to 44°. This paper shows that organisms of other genera become nutritionally more exacting on increase in growth temperature.

METHOD

The following modification of Gale's (1947) medium devised by Bigger (1950) was used: Glucose, 3·0 g.; $(NH_4)_2HPO_4.2H_2O$, 5·0 g.; NaCl, 1·0 g.; $FeSO_4.7H_2O$, 0·3 g.; $MgSO_4.7H_2O$, 0·7 g.; KH_2PO_4, 0·5 g.; K_2HPO_4, 5·0 g.; water to 1000 ml.; pH value adjusted to 7·4.

The substances added to the medium were dissolved in distilled water, adjusted to pH 7·4, sterilized by boiling and added to the medium to give the final concentration required. The test organisms were grown in the basal medium for 24 hr. at 37° and the inoculum prepared by dilution in buffered saline. Five ml. quantities of the test medium were inoculated with 1 drop of the inoculum from a Pasteur pipette which delivered 22 drops/ml. An incubator was used for 37° and below and a water-bath having a stability ±0·2° for higher temperatures. The alternative methods of incubation did not influence the result.

RESULTS

The effect of temperature on the growth requirements of Proteus vulgaris

Our strain of *Proteus vulgaris* grew well in Lab-lemco broth between 25° and 44°. In the test medium growth occurred between 25° and 40° only when 5 mg. nicotinamide/100 ml. was added, and was enhanced by the further addition of one of several nitrogenous substances (Table 1). At 41° addition of any one of a number of these substances was essential for growth; at 42° only four of these compounds were capable of supporting growth, namely: L-glutamic acid, DL-methionine, L-cystine, thiamine; and at 43° only L-glutamic acid and DL-methionine; at higher temperatures growth did not occur. The temperature at which growth occurred was limited in some instances by the concentration of the added substance; L-leucine, however, was inhibitory in high concentration.

Table 1. *The effect of various substances on the growth of* Proteus vulgaris *and of* Salmonella typhi *in a defined medium at various temperatures*

The amounts of growth were read at 48 hr. for *Proteus vulgaris* and at 24 hr. for *Salmonella typhi.*

	P. vulgaris							*S. typhi*	
	Temperature of growth								
	40°	41°		42°		43°		40°	
	Concentration (mg./100 ml.)								
Substance added to defined medium	0·01	10–100	1·0	10–100	1·0	10–100	1·0	10–100	1·0
L-Alanine	X	0	0	0	0	0	0	0	0
L-Arginine	X	X	X	0	0	0	0	X	X
L-Asparagine	X	X	X	0	0	0	0	0	0
L-Aspartic acid	sl.	X	0	0	0	0	0	0	0
L-Cystine	X	X	X	X	X	0	0	0	0
L-Glutamic acid	X	X	X	X	X	X	0	X	X
Glycine	sl.	0	0	0	0	0	0	0	0
L-Histidine	X	X	X	0	0	0	0	0	0
L-Isoleucine	X	X	X	0	0	0	0	0	0
L-Leucine	X	0	0	0	0	0	0	0	0
L-Lysine	X	X	X	0	0	0	0	X	X
DL-Methionine	X	X	X	X	X	X	0	0	0
Nicotinamide	—	—	—	—	—	—	—	0	0
Thiamine	X	X	X	X	0	0	0	X	0
DL-Tryptophan	X	X	X	0	0	0	0	0	0
L-Valine	X	0	0	0	0	0	0	0	0
Controls									
Defined medium + 5 mg./100 ml. nicotinamide	sl.	0		0		0		0	
Defined medium	0	0		0		0		0	
Lemco broth	X	X		X		X		X	

0 = no turbidity; X = turbidity; sl. = slight turbidity.

The effect of temperature on the growth requirements of Salmonella typhi

The growth of our strain of *Salmonella typhi* in 24 hr. at 37° in the test medium was enhanced by the addition of any one of a number of nitrogenous substances in concentrations of from 1 mg./ml. to 1 mg./100 ml. The only substance which had an adverse effect on growth was nicotinamide. There was no growth after incubation at 40° for 24 hr. unless one of the following substances was added: L-glutamic acid, thiamine, L-lysine, L-arginine. At 43° no single addition tested was able to support growth, but L-glutamic acid + L-arginine with either thiamine or L-lysine showed growth in 24 hr.; without L-arginine growth was delayed for 48 hr. The addition of DL-methionine either to L-glutamic acid + L-lysine or to L-glutamic acid + thiamine inhibited growth, but there was no inhibition when DL-methionine was added to L-glutamic acid + thiamine + L-lysine; thiamine or L-lysine could, in this combination, be replaced by L-arginine which also overcame the inhibitory effect of DL-methionine. L-Glutamic acid was always essential for growth. Although active at 10 mg./100 ml. the optimum concentration of each substance was approximately 200 mg./100 ml.

DISCUSSION

Hills & Spurr (1952) and Ware (1951) suggested that increase in incubation temperature caused changes in certain nutritional enzyme systems; it appears that enzymes responsible for L-glutamic acid formation may be among the first to be affected by increased temperature.

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Genetic Factors Influencing the Activity of Tryptophan Desmolase in *Neurospora crassa*

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SUMMARY: A simple method for purification of tryptophan desmolase from *Neurospora crassa* in a good yield is described. No difference could be detected between the tryptophan desmolase extracted from a back-mutated strain of mutant C 83 and that from the wild-type strain. Michaelis constants with respect to the substrates indole and serine and the co-factor pyridoxal phosphate were measured as the basis for possible differences. It is shown that the concentration of tryptophan desmolase in the organism can be determined by genes other than those which induce a tryptophan requirement. Crosses of a histidine-requiring mutant to wild type yielded mutant progeny with 2 to 3 times the normal activity and progeny with no detectable activity under certain conditions. The lack of activity appears to be due to the production of an inhibitor.

Mitchell & Lein (1948) reported one of the first examples in *Neurospora crassa* in which the loss of activity of an essential enzyme was directly attributable to a gene mutation. This case concerned a *N. crassa* mutant (C 83) which has an L-tryptophan requirement that cannot be satisfied by indole. The enzyme activity which was lost as a result of this mutation was that of tryptophan desmolase, the enzyme in *N. crassa* which catalyses the synthesis of L-tryptophan from indole and L-serine (Umbreit, Wood & Gunsalus, 1946; Yanofsky, 1952*a*). At a later date it was thought that tryptophan desmolase activity had been found in the C 83 mutant (Gordon & Mitchell, 1950), but this observation could not be reproduced and has been retracted (Horowitz & Mitchell, 1951). Yanofsky (1952*b*) also reported that he could not detect this enzyme activity in mutant C 83.

The simple picture of the genetic factors responsible for tryptophan desmolase activity in *Neurospora crassa* has recently been complicated by the findings of Yanofsky (1952*b*) who reported a mutant, S 1952, which appears to be allelic to mutant C 83 and has the same growth requirements and inability to produce detectable amounts of tryptophan desmolase activity. However, when a particular suppressor gene is introduced into strain S 1952, detectable amounts of tryptophan desmolase are produced, whereas the mutant C 83 in combination with the same suppressor gene does not produce detectable amounts of this enzyme.

The present paper deals with two different gene changes in *Neurospora crassa* which influence the tryptophan desmolase activity. One of these, the back mutation of mutant C 83, fits into the original simple picture, whereas the other, a gene mutation responsible for an L-histidine growth requirement, C 84 (Haas, Mitchell, Ames & Mitchell, 1952), tends to complicate the picture. It is the purpose of this paper to characterize the changes in tryptophan desmolase activity associated with each of these genetic changes and to analyse their significance.

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METHODS

Crude extracts. Specific activity determinations of tryptophan desmolase in crude extracts were used as a measure of the amount of tryptophan desmolase present in the strains relative to the total amount of protein present. Several wild-type stocks and re-isolates were tested. These and the mutants used were derived from a number of wild stocks (Beadle & Tatum, 1945). The crude extracts were prepared in the following manner.

The organism was grown at 25° in 125 ml. flasks containing 20 ml. minimal medium (Beadle & Tatum, 1945) supplemented with the appropriate growth factor. At the desired time, the mycelial pads were removed and washed once with distilled water. One g., wet weight, of mycelium was ground for 5 min. in a mortar (13 cm. in diameter and 7 cm. high) containing 0·5 g. sand, 2 ml. 0·2 M-K_2HPO_4 containing 2 mg. DL-serine/ml. (to stabilize the enzyme), and 2 ml. 0·2 M-potassium phosphate buffer (pH 7·7). The ground material was centrifuged for 20 min. in a Servall centrifuge at *c.* 5000 r.p.m. and the supernatant fluid used as the crude extract. No appreciable activity could be found in the centrifuged precipitates and the specific activity of the crude extracts did not change with increased grinding time up to 25 min.

Measurement of tryptophan desmolase activity. Solutions of indole, serine and pyridoxal phosphate were placed in a 6 in. test tube and equilibrated to 37° in a water-bath. At time $t=0$, the appropriate amount of tryptophan desmolase extract (also equilibrated to 37°) was added and the mixture stirred for a few seconds. Samples (1.0 ml.) were removed at convenient intervals and analysed for indole by a modification of the method of Fearon (1944). Thus the 1·0 ml. samples were immediately mixed with 5·0 ml. of a solution of 1·0 g. xanthydrol/l. glacial acetic acid. The mixture was then heated at 100° for 30 min., cooled, and the intensity of the colour produced determined with a Klett-Summerson colorimeter equipped with a green filter (transmission range *c.* 5000–5700 A). The initial velocity (v_0) of the reaction was determined from a plot of colorimeter readings versus time; generally four determinations were used for each plot. The specific activity of the extract (mμmole/min./mg. protein-nitrogen) is this initial velocity multiplied by the dilution factor of the extract in the reaction mixture and divided by the protein-nitrogen content of the extract. Activity determinations which were used as a measure of the amount of tryptophan desmolase present in an extract were determined under the following standard initial conditions: temperature = 37·0°; 0·2 M-potassium phosphate buffer (pH 7·6); $4{\cdot}2 \times 10^{-4}$ M-indole; 0·17 M-DL-serine; $4{\cdot}8 \times 10^{-5}$ M-calcium pyridoxal phosphate. Under these conditions, the v_0 is proportional to the concentration of the enzyme, and for a given enzyme concentration, v_0 does not increase significantly when the concentrations of the substrates and pyridoxal phosphate are doubled. DL-Serine was used here in place of L-serine, since the same initial velocities are observed under these conditions whether 0·17 M-DL-serine or 0·085 M-L-serine is employed. The protein-nitrogen content of the extracts was determined by micro-Kjeldahl digestion of 5% trichloroacetic acid precipitates, followed by Nesslerization.

Partial purification of tryptophan desmolase. Large amounts of mycelium were grown at 25° in 8 or 16 l. of minimal medium under forced aeration. After growth for 4 days, the mycelium was removed, washed 3 times with distilled water, and used immediately or stored at −16°. Fifteen g. mycelium were ground for 10 min. in a mortar containing 7·5 g. sand and 15 ml. 0·2 M-K_2HPO_4 containing 2 mg. DL-serine/ml. This ground material was centrifuged for 20 min. in a Servall centrifuge at 5000 r.p.m. and the supernatant fluid retained. This crude extract was heated at 54° for 6 min. and cooled to 0°. This heat treatment stabilized the tryptophan desmolase, yielding a preparation whose first-order inactivation constant at 3° was one-third that of the untreated preparation. The extract was then centrifuged in a Spinco Model L ultracentrifuge for 45 min. at 40,000 r.p.m. (145,000 g maximum). The precipitate was discarded and 18·7 g. solid $(NH_4)_2SO_4$/100 ml. extract added and the mixture stirred for 40 min. The extract was then centrifuged at 5000 r.p.m. for 20 min. in a Servall centrifuge. The supernatant fluid was discarded and the precipitate washed once with a solution of 19·1 g. $(NH_4)_2SO_4$/100 ml. 0·2 M-potassium phosphate buffer (pH 7·7), and the precipitate taken up in 0·2 M-potassium phosphate buffer (pH 7·6), to yield the partially purified material. Unless otherwise indicated, all operations were carried out at 0−3°. This purification procedure yielded a clear solution with a specific activity 15–20 times greater than that of the crude extract (e.g. in one experiment, specific activity of crude and partially purified extracts were 19 and 320 mμmole/min./mg. protein-nitrogen, respectively); the average yield was approximately 60%. This procedure gives about the same purification as that of Yanofsky (1952*a*), but has the advantage of being simpler and of giving higher yields.

RESULTS

Tests for tryptophan desmolase in the C 83 mutant

Experiments designed to detect tryptophan desmolase activity in the C 83 mutant were without success. Mixture of the following extracts from C 83 with active wild-type preparations did not alter the activity of the wild-type extracts: washed mycelium, crude extract, dialysed extract, cell debris, extracts purified according to enzyme purification procedure and precipitates resulting from addition of various amounts of $(NH_4)_2SO_4$ to crude extracts. This indicated that no inhibitor was present in the C 83 extracts. Yanofsky (1952*b*), using similar conditions and methods, was also unable to find any activity in this mutant.

In order to obtain evidence that the tryptophan requirement and the deficiency of tryptophan desmolase in strain C 83 are due to the same gene change, a cross of the mutant C 83 (mating type A) to wild type 8 (mating type a) was made and asci were dissected. An examination of 19 asci revealed that there was a 1:1 segregation of these two characteristics and that they always appeared together. With one exception, the specific tryptophan desmolase activity of each of the wild-type progeny did not differ significantly from that of the parent strain (8a; specific activity = 17). This exception concerned one wild-type spore pair which in six separate experiments gave specific activities of 0, 0, 0, 13, 17, and 15 when grown for 4 days in minimal medium containing 5×10^{-4} M-L-tryptophan. It has been demonstrated that the anomalous zero specific activities found for this strain result from the fact that when grown in the presence of L-tryptophan (but not in its absence) this strain loses enzyme activity at some time between 3 and 5 days of growth, although it contains normal enzyme activity at earlier stages of growth. This strain is not heterocaryotic with respect to the mutant gene of C 83 and thus this effect of L-tryptophan on the enzyme activity cannot be explained by a preferential multiplication of C 83 nuclei over wild-type nuclei. Although a complete genetic analysis of the character has not been made, it is clear that the phenomenon is independent of the C 83 mutation. This does not however exclude the possibility that the influence of L-tryptophan may be operative in the C 83 mutant.

The back mutation of C 83

A triple mutant of C 83, C 102 and the albino 15300 prepared from the single mutants by crossing was employed in the experiments concerning the back mutation of C 83, the C 102 and 15300 mutant characters being used as markers. The C 102 mutant is phenotypically wild type at 25° but 'colonial' at temperatures above 31° (Mitchell & Mitchell, 1952); this has the advantage of making viable conidial counts relatively easy. As was indicated previously, it was impossible to find any evidence for the existence of tryptophan desmolase in the C 83-C 102-15300 strain.

The back mutation of C 83-C 102-15300 was accomplished in the following manner: a glass-wool filtered suspension of C 83-C 102-15300 macroconidia containing $3{\cdot}1 \times 10^6$ viable conidia/ml. was divided into two equal parts, and one of these was subjected to ultraviolet irradiation from a Model UV-30 American Sterilizer Company sterilizing lamp for 2 min. at a distance of 10 cm. The other half was not irradiated and was used as the control. The irradiated material contained $1{\cdot}6 \times 10^6$ viable conidia/ml., representing a survival of 52%. Thirty 125 ml. flasks, each containing 20 ml. minimal medium, were then inoculated with 0·1 ml. of the irradiated suspension ($1{\cdot}6 \times 10^5$ viable conidia/flask) and twenty-eight such flasks were inoculated with 0·1 ml. of the control suspension ($3{\cdot}1 \times 10^5$ conidia/flask). These flasks were then allowed to stand at 25° and were observed over a period of 15 days. Twenty-seven of the thirty flasks inoculated with irradiated conidia contained appreciable amounts of mycelium by this time, whereas none of the flasks inoculated with non-irradiated conidia showed any growth. Thus no reversions were noted in a total of 9×10^7 viable conidia not subjected to irradiation, whereas at least one reversion occurred for every 2×10^5 viable conidia subjected to irradiation. Subcultures from those flasks in which growth occurred all exhibited the characteristics of the C 102-15300 double mutant and were indistinguishable from wild type in the amount of growth achieved in four days on minimal medium.

That this reversion of C 83 to wild type was indeed of genetic origin was demonstrated in the following manner. A cross of a reverted culture, C 83re-C 102-15300a to C 83-C 102-15300A was made, and the resulting progeny were examined for the 3 mutant characters, tryptophan requirement being used in the case of C 83. Considering only one ascus/perithecium, all twenty asci examined carried the albino 15300 and 'colonial' C 102 markers. Nine of these asci gave a spore pattern of 4 tryptophan-independent (T^+) and 4 tryptophan-dependent (T^-) spores, while in the remaining 11, all 8 spores were T^-. An examination of 2402 random spores germinating on minimal agar revealed 614 (26 %) which had a wild-type germination pattern and 1788 (74 %) which had a germination pattern identical with the C 83 mutant. These results are those expected if one considers that the macroconidia originally irradiated generally contain more than one nucleus, and hence the original reverted cultures would be expected to be heterocaryotic for T^+ and T^- (Giles, 1951). One would thus expect to find both the $4T^+$:$4T^-$ and the $8T^-$ spore patterns in the asci resulting from the above cross, the frequency of each type presumably depending upon the frequency with which the T^+ and T^- nuclei occur in the heterocaryon.

That this genetic reversion to wild type represents a back mutation and not the action of some suppressor gene was demonstrated in the following manner. A cross of wild type P 1347a with C 83re-C 102-15300A, a homocaryotic reverted strain isolated from the progeny of the previous cross, was made and forty asci were dissected. All of these asci had $8T^+$ spore patterns. Furthermore, in an examination of 8791 random ascospores, no T^- types were found.

The homocaryotic back-mutated strain C 83re-C 102-15300 was compared with the corresponding strain, C 102-15300, for growth and tryptophan desmolase characteristics. The growth-time curves for these two strains when grown on minimal medium or minimal plus 5×10^{-4} M-L-tryptophan are given in Fig. 1. The two curves are typically wild type (e.g. strain 8a), and it is apparent that there was no significant difference between the two strains. No specific difference in the tryptophan desmolase from the two strains could be detected. Crude extracts from the two strains were partially purified (see Methods) and the tryptophan desmolase in the two preparations examined. As can be seen from Table 1, no significant differences in the kinetic constants (K_M values) or the pH optimum exist between the two preparations.

Taken collectively, these data indicate that the allele of the C 83 gene which results from the back mutation of C 83 is the same as the wild-type allele from which C 83 was derived. At present the C 83 mutation must thus be considered as an essentially reversible process which is accompanied by a loss or gain of detectable tryptophan desmolase activity.

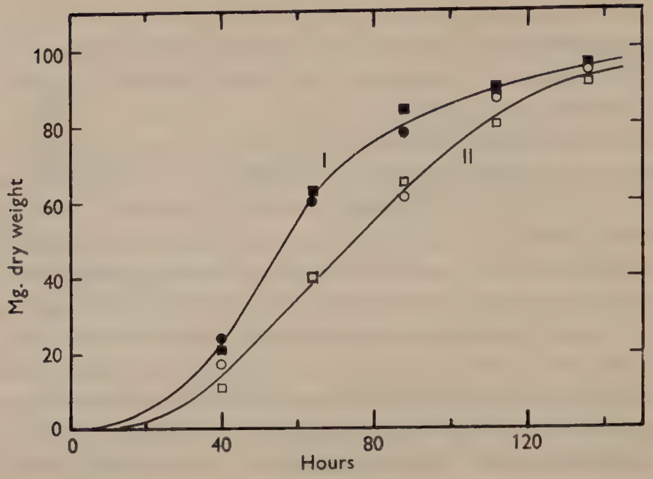

Fig. 1. Growth curves for the back-mutated strain C 83re-C 102-15300 (squares) and strain C 102-15300 (circles) on minimal medium (I) and on minimal medium + 5×10^{-4} M-L-tryptophan (II).

Table 1. *Properties of tryptophan desmolase in partially purified extracts from strains C* 102-15300 *and C* 83*re-C* 102-15300 *of* Neurospora crassa

	Strains	
Property	C 102-15300	C 83re-C 102-15300
Michaelis constants, K_M*		
Indole	$2{\cdot}3 \times 10^{-5}$ M	$2{\cdot}8 \times 10^{-5}$ M
L-Serine	$6{\cdot}3 \times 10^{-3}$ M	$5{\cdot}7 \times 10^{-3}$ M
Pyridoxal phosphate	$3{\cdot}4 \times 10^{-6}$ M	$4{\cdot}4 \times 10^{-6}$ M
pH optimum	7·6	7·6

* Determined at 37° in 0·2 M-phosphate buffer (pH 7·6) by Lineweaver-Burk plots (Lineweaver & Burk, 1934) of the reciprocals of the v_0 values versus the reciprocals of the initial concentrations, five different concentrations being used for each plot. The accuracy of these K_M values is approximately ±15 %. The initial concentrations employed were as follows:

	Initial concentration (M)		
K_M determined	Indole	L-Serine	Pyridoxal phosphate
Indole	$0{\cdot}47$ to $4{\cdot}7 \times 10^{-4}$	$8{\cdot}0 \times 10^{-2}$	$4{\cdot}6 \times 10^{-5}$
L-Serine	$4{\cdot}7 \times 10^{-4}$	$3{\cdot}8 \times 10^{-3}$ to $8{\cdot}0 \times 10^{-2}$	$4{\cdot}6 \times 10^{-5}$
Pyridoxal phosphate	$4{\cdot}7 \times 10^{-4}$	$8{\cdot}0 \times 10^{-2}$	$0{\cdot}39$ to $4{\cdot}6 \times 10^{-5}$

The change in tryptophan desmolase activity attendant on the mutation to C 84

The C 84 mutant has an L-histidine requirement that cannot be satisfied by any other compound so far tested and is associated with a single gene (Haas *et al.* 1952). A comparison of the tryptophan desmolase specific activity in extracts of this strain with that of wild type 8a when both are grown on minimal medium supplemented with $3{\cdot}2 \times 10^{-4}$ M-histidine is shown in Fig. 2 along with the growth curves of the two strains. The two- to threefold greater specific activity observed in the C 84 extracts after 50 hr. of growth apparently is not the effect of either an inhibitor in the wild type or an activator in the C 84, since mixtures of the two extracts had activities which were the sum of their individual activities. Furthermore, dialysis of these extracts did not alter their activities.

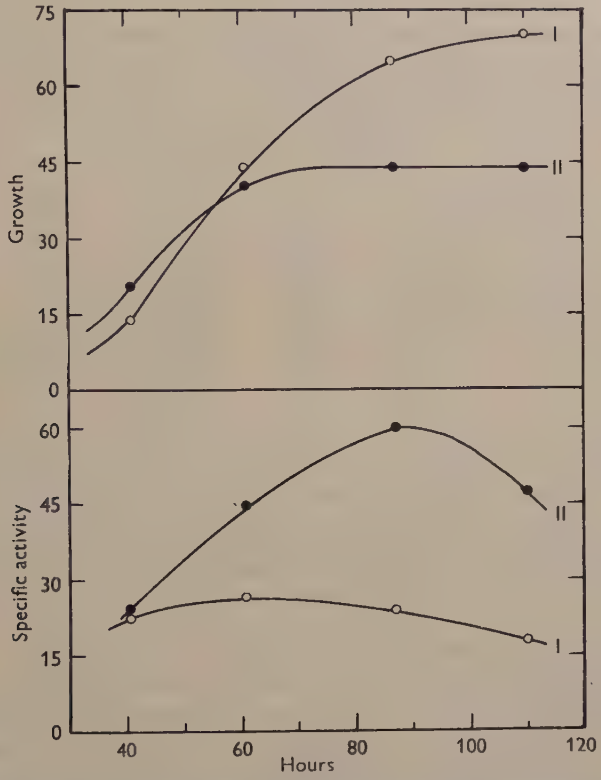

Fig. 2. Growth (mg. dry weight/flask) and specific activity (mμmole/min./mg. protein-nitrogen) as a function of time of wild type (I) and mutant C 84 (II). These strains were grown at 25° in minimal medium + $3{\cdot}2 \times 10^{-4}$ M-L-histidine.

In order to determine whether or not the high specific activity of the C 84 strain was directly associated with the histidine requirement of this strain, a cross of C 84A with wild type 8a was made and the progeny examined for histidine requirement and tryptophan desmolase activity. The results from the nine asci that were examined are given in Table 2. All of the L-histidine-independent (H^+) strains are seen to possess specific activities which do not differ significantly from the wild-type parent. However, the L-histidine-dependent (H^-) strains are seen to possess specific activities which can roughly be classified in two groups: low specific activity, 0–3·7 mμmole/min./mg. protein-nitrogen; high specific activity, 32–60 mμmole/min./mg. protein-nitrogen, which is similar to that of the C 84 strain. Using this classification, the nine asci fall into three groups: (1) five asci with $4H^+$ wild-type activity: $4H^-$ low activity; (2) two with $4H^+$ wild-type activity: $2H^-$ low activity: $2H^-$ high activity; (3) two with $4H^+$ wild-type activity: $4H^-$ high activity.

Were there a direct association between the histidine requirement and the high activity of the C 84 parent strain, all of the asci would be $4H^+$, wild-type activity: $4H^-$, high activity. Thus it is the presence of the H^-, low activity progeny which disproves this association.

Table 2. *Specific activity of tryptophan desmolase in crude extracts from the parents and progeny of C* 84*A* × 8*a**

Parents: Specific activity of 8a = 17; specific activity of C 84A = 47.

		Specific activity for spore pairs†			
Progeny:	Ascus no.	H^-	H^-	H	H
	1	**0·0**	2·2	**18**	19
	2	0·0	1·9	**16**	18
	3	**3·7**	0·0	**19**	**15**
	4	**0·0**	**0·0**	17	**16**
	5	0·0	**0·0**	**17**	**16**
	6	**0·0**	43	**22**	**22**
	7	0·0	**42**	**18**	16
	8	33	**45**	**17**	**22**
	9	35	60	**16**	**17**

* The units of specific activity are mμmole/min./mg. protein-nitrogen. The specific activities of the parent strains are the average of 5 independent determinations. Crude extracts were obtained from mycelia grown for 4 days at 25° in minimal medium + $3{\cdot}2 \times 10^{-4}$ M-L-histidine.

† The specific activities given in **black** are the average of the specific activities of the members of the spore pair. There was never any significant difference between the members of a pair. In the other cases only one member of each spore pair was tested.

The H^- low activity progeny are all the more remarkable in that, while apparently containing no tryptophan desmolase activity, they grew as well as the H^- high activity progeny in the absence of exogenous L-tryptophan, both types of H^- progeny having the same growth curve as the C 84 parent. All of these strains, along with the H^- high activity and the H^+ progeny, were therefore examined for tryptophan desmolase activity at various stages of growth; the results are shown in Fig. 3. The wild type and the H^-, high activity progeny exhibit the same specific activity-time curves as the wild type 8a and H^- C 84A parents, respectively. The extracts of the H^-, high activity and wild-type progeny are also like the extracts from the parent strains in that mixtures of the two have activities which are the sum of their individual activities. Although the activity present in the H^- low activity progeny before 50 hr. explains how these strains can grow in the absence of exogenous L-tryptophan, it is difficult to imagine that the succeeding fall and rise of specific activity represents a rapid destruction followed by a rapid synthesis of tryptophan desmolase. An inhibitor of tryptophan desmolase catalysis was therefore sought in the extracts which showed low activity.

Experiments which used mixtures of active extracts from wild type 8a and inactive extracts from the H^- low activity progeny indicated that an inhibitor was indeed present in the inactive extracts. Thus the representative data in Table 3 show that, as the ratio of inactive extract to active extract is increased, the ratio of the tryptophan desmolase activity observed to that expected if the inactive extracts were inert to the tryptophan desmolase system, decreased. This decrease is probably due to an inhibitor and not to destruction of the enzyme, since the activity of the mixed extracts decreased with time at approximately the same rate as that of the active extract alone.

Although it is apparent that the inactive extracts contained a tryptophan desmolase inhibitor, whether or not these extracts contain tryptophan desmolase has not been determined. The rapid rise in activity after 100 hr. of growth (Fig. 3) would indicate that they probably do, but a definite answer to this question will have to await the results of investigations as to the nature of this inhibitor.

Table 3. *Inhibition of tryptophan desmolase in wild-type extracts by inactive extracts of an H^- low activity strain from C 84 A × 8 a cross*

Fraction wild-type extract (8a)	Fraction inactive extract	Observed activity*	Calculated activity†	Observed activity / Calculated activity
1·00	0·00	3·3	3·3	1·00
0·76	0·24	2·2	2·5	0·88
0·50	0·50	1·0	1·6	0·62
0·24	0·76	0·1	0·8	0·12
0·00	1·00	0·0	0·0	—

* Activity is expressed in mμmole/ml./min.

† Calculated on the assumption that the inactive extract is inert relative to the tryptophan desmolase activity of the wild-type extract.

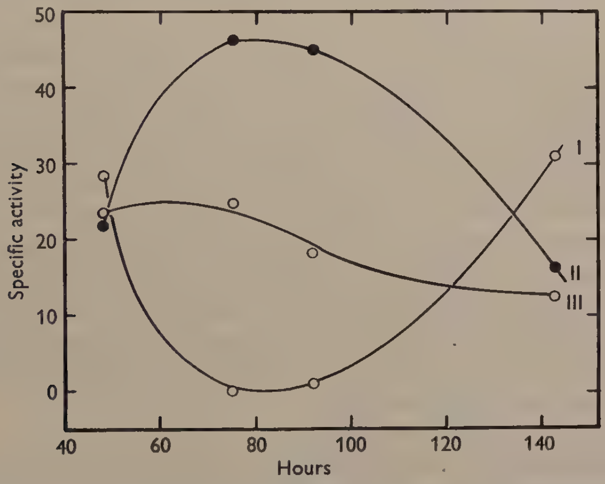

Fig. 3. Specific activity (mμmole/min./mg. protein-nitrogen) as a function of time of growth of the three types of progeny from C 84 A × 8 a. I = H^-, low activity. II = H^-, high activity. III = H^+ (wild type). These strains were grown at 25° in minimal medium + $3{\cdot}2 \times 10^{-4}$ M-L-histidine.

It is apparent that the data on the cross of C 84 A to 8 a cannot at present be used to evaluate the quantitative effect of the gene responsible for the histidine dependence of C 84 on the actual amount of tryptophan desmolase present in the mould. However, since all the wild-type progeny of this cross have the activity of the wild-type parent, it is very probable that this mutant gene (or one closely linked to it) can influence the activity of the enzyme. It is of course possible that one or more specific genes must be present with the C 84 gene in order to alter the activity of tryptophan desmolase, but the uniform parent wild-type activity of the H^+ progeny would indicate that the other gene or genes could not influence this activity level except in combination with the C 84 gene.

DISCUSSION

The original observations on mutant C 83 (Mitchell & Lein, 1948), those by Yanofsky (1952*a*, *b*) and the ones described here are consistent in the demonstration that this mutant does not contain a detectable quantity of tryptophan desmolase when grown under a variety of conditions. These data are in accord with the simple hypothesis that the wild-type allele of the mutant gene of C 83 has a direct function in controlling the synthesis of tryptophan desmolase. However, as shown by the studies on the activity of this enzyme in extracts of the histidine-requiring mutant (C 84) and by the limited information on one wild-type segregant from a C 83 × wild type cross, a number of factors can affect the degree of activity; indeed under certain conditions these strains are very similar to the C 83 mutant in that they contain no detectable activity. In this connexion it is of interest to note that L-tryptophan, indole and certain methyl analogues of these compounds inhibit the formation of tryptophan desmolase activity in wild type *Aerobacter aerogenes* (Cohn & Monod, 1953). That this effect by tryptophan can exist to some extent, at least in *Neurospora crassa*, has been shown here and since the strain C 83 will not grow in the absence of tryptophan it is not possible to evaluate the influence of these required growth conditions on the degree of enzyme activity in the mutant. It does seem clear in the case of the low degree of activity in the histidine mutant that an inhibitor of enzyme catalysis is involved, while there is no evidence that this is the case in C 83. Nevertheless, an *in vivo* inhibition of enzyme formation in C 83 is not excluded and available data do not reveal the reasons why this strain is deficient in tryptophan desmolase activity or by what mechanism the wild-type allele is functional in determining the presence of the enzyme activity. Strauss (1953) observed that a *N. crassa* mutant which had a low degree of carboxylase activity had associated with it a low degree of tryptophan desmolase activity.

It is of special interest to note that the tryptophan desmolase derived from a back-mutated strain of C 83 was not significantly different from that obtained from the parental strain with respect to the apparent substrate and co-factor Michaelis's constants. Yanofsky (1952*b*) made similar observations with an enzyme preparation from *Neurospora crassa* strain S 1952 in which enzyme activity had been restored by action of a suppressor gene. The mutant gene of S 1952 is either allelic with or very closely linked to that of C 83, while the suppressor gene is at a different locus but it does not act to restore tryptophan desmolase activity in strain C 83, as it does in S 1952 (Yanofsky, 1952*b*). It is clear from these observations that restoration of what appears to be the same enzyme can occur either through back-mutation or by action of an independent suppressor gene. Again these results do not distinguish between direct and indirect action of a gene in determining the presence or absence of an active enzyme.

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The Effect of certain Enzyme Inhibitors on Respiration and on Penicillin Formation by *Penicillium chrysogenum*

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SUMMARY: The effect of phenol, mercuric chloride, fluoride, bisulphite, azide, malonate, 2:4-dinitrophenol and cyanide on respiration and penicillin production was studied using suspensions of washed penicillin-producing mycelium of *Penicillium chrysogenum* in Warburg flasks. Inhibition of penicillin production by phenol and by mercuric chloride closely followed the inhibition of respiration. Both fluoride and bisulphite inhibited penicillin production to a greater extent than respiration. Malonate was without effect on respiration or penicillin production, possibly because the pH was too high to allow a sufficient concentration of undissociated acid. Penicillin production was inhibited by 2:4-dinitrophenol at concentrations which had little effect on respiration, suggesting that phosphate bond energy is utilized in penicillin formation. Penicillin production was extremely sensitive to cyanide. A concentration of 0·000002 M-KCN, which caused no significant inhibition of respiration, depressed the rate of penicillin production to *c.* 25 % of normal. Maximum rates of penicillin production and respiration are dependent on cyanide-sensitive systems but an alternative, relatively cyanide-stable system allows both respiration and penicillin production to proceed at *c.* 25 % of the maximum rate.

Despite a continuing increase in knowledge of the fermentation conditions necessary to obtain high penicillin yields, virtually nothing is known of the mechanism, or path, of synthesis of penicillin. It is well known that the side chain of the penicillin molecule can be supplied in the form of a precursor such as phenylacetic acid, the benzyl group of which is incorporated into the molecule of penicillin G; but no successful attempts to supply precursors for other parts of the molecule have been reported. Sulphur is a constituent of the molecule and organic sulphur-containing compounds such as L-cystine, L-cysteine, methionine and glutathione appear to be utilized by the mould in preference to sulphate for penicillin formation (Stevens, Vohra, Inamine & Roholt, 1953). So far as I am aware, no intermediate compounds in the pathway of penicillin synthesis have been identified and nothing is known of the enzymes involved. Experiments were therefore carried out with certain enzyme inhibitors in an attempt to throw some light on the mechanism of penicillin formation.

METHODS

The experiments were carried out with washed penicillin-producing mycelium suspended in a medium of known composition in Warburg flasks. The penicillin produced after 1½–2 hr. was then assayed by the biological cup-plate method in the usual way. This technique enabled penicillin production to be studied as a reaction under conditions which changed very little during an experiment and which could be controlled in a way impossible in a complete fermentation.

The samples of mycelium used were obtained from highly aerated submerged fermentations during the period of active penicillin production. Mycelium was washed by repeated filtration on a Buchner funnel to remove penicillin and original medium, and was finally suspended in the medium to be used in the experiment. It was necessary to repeat the washing at least 3 times to ensure that the penicillin concentration in the suspension was <2 units/ml.

The washing of the mycelium, which might take up to 30 min., did not impair its penicillin-producing ability. With adequate agitation, washed mycelium suspended in a medium similar to that used in the fermentation from which it was obtained, formed penicillin at a rate at least equal to that in the unaltered fermentation itself. Two ml. volumes of suspension were used in each Warburg flask, maintained at 26°.

For maximum rates of penicillin production it was essential that the rate of oxygen uptake by the mycelium should not be limited by the rate of solution of oxygen in the Warburg flasks. Agitation of the flasks at 200 strokes/min. with a 5 cm. stroke was required to provide an adequate rate of solution for a suspension of mycelium of density equivalent to 0·7 g. dry-wt. mycelium/100 ml. (0·7 %). With the Warburg apparatus used, adequate aeration could not be provided for suspensions of density 1·0 % or greater. The oxygen demand of the mycelium, and hence the degree of agitation required, varies with the age of the mycelium, the conditions under which it is grown, and the composition of the medium used for suspending. In all these experiments the mycelium used was of a standard age having been grown under standard fermentation conditions in a lactose + corn steep liquor + salts medium. The organism used was a mutant which did not form pigment, derived from *Penicillium chrysogenum* strain W 48–701. The medium used to wash and suspend the mycelium contained: corn steep liquor, 2 % (v/v); lactose, 3 % (w/v); $NaNO_3$, 0·3 %; KH_2PO_4, 0·05 %; Na_2SO_4, 0·1 %; phenylacetic acid, 0·02 %; pH value 6·0–6·2. It was boiled for 1–2 min. to destroy any penicillinase in the corn steep liquor and then filtered. With this medium, penicillin production occurred at a uniform rate in Warburg flasks during at least 3 hr. That aeration was adequate was confirmed in every experiment either by rocking the manometers at a lower rate for a short period of time to demonstrate that the rate of oxygen uptake was not limiting, or by carrying out the whole experiment in duplicate with two suspensions of different mycelial densities.

Despite the relatively brief duration of the experiments, some growth (usually *c.* 10 %) did occur, with a corresponding increase in the rate of oxygen uptake. At the end of the experiment the contents of the flasks were washed out with 0·05 M-KH_2PO_4 + NaOH buffer (pH 7·0) and made up to standard volume before assay. Rupture of the cell walls of washed mycelium by shaking with glass beads in a Mickle disintegrator and assay of the penicillin liberated showed that the penicillin within the mycelium at the beginning of an experiment could contribute <2 units/ml. to a normal suspension by outward diffusion during the experiment.

The presence of enzyme inhibitors did not interfere with the assay of the penicillin content of the suspensions. The original 2 ml. of suspension in the

Warburg flasks could usually be diluted at least 3 times before assay so that at the maximum concentration of each inhibitor used, none showed any inhibition on the assay plates when tested separately nor did they alter the inhibition zone diameters of standard penicillin solutions.

In the experiments with cyanide, suitable mixtures of KCN and KOH were used (Umbreit, Burris & Stauffer, 1945) in the centre well of the Warburg flasks to prevent cyanide distilling over from the suspension into the KOH in the centre well and so altering the concentration of cyanide during the experiment.

RESULTS

Figures for oxygen uptake and penicillin production are given as percentages of those obtained in controls without inhibitor.

Effects of phenol and mercuric chloride

Phenol and mercuric chloride were chosen to determine the effect of relatively non-specific poisons; the results are shown in Fig. 1. In low concentrations, phenol appeared to stimulate oxygen uptake slightly without any corresponding stimulation of penicillin production. Concentrations >0·002 M, however, caused a similar degree of inhibition of both oxygen uptake and penicillin production so that although the curve for respiration was displaced upwards by the slight stimulatory effect of phenol it was essentially parallel to that for penicillin production. In this case the effect on penicillin production is probably indirect, as a result of the effect on the mycelium as a whole. The effect of mercuric chloride on penicillin production is also paralleled by its effect on total oxygen uptake.

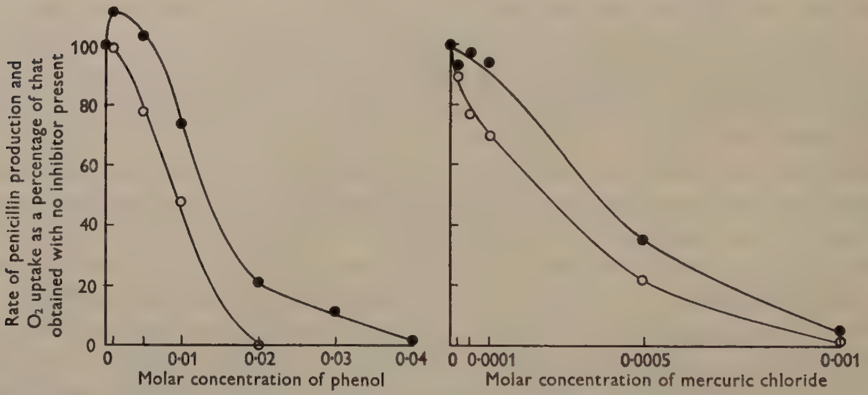

Fig. 1. Effect of phenol and mercuric chloride on penicillin production (○——○) and oxygen uptake (●——●) by *P. chrysogenum*.

Fluoride and bisulphite

As shown in Table 1, both fluoride and bisulphite inhibited penicillin production to a greater extent than total oxygen uptake. Sodium fluoride at 0·02 M had little effect on respiration but penicillin production was *c.* 50 % of maximum; with 0·08 M respiration was markedly depressed, and no penicillin was detected. With bisulphite, a concentration of 0·01 M depressed oxygen uptake to 69 % of maximum and penicillin production to 24 % of maximum.

Table 1. *Effect of sodium fluoride and sodium bisulphite on penicillin production and oxygen uptake by* Penicillium chrysogenum

Rates of penicillin production and oxygen uptake as percentage of that obtained without inhibitor. Concentrations of sodium bisulphite up to 0·02 M showed no significant oxygen uptake during the period of taking manometer readings as a result of oxidation to sulphate.

Sodium fluoride			Sodium bisulphite		
Conc. (M)	Rate of O_2 uptake (%)	Rate of penicillin production (%)	Conc. (M)	Rate of O_2 uptake (%)	Rate of penicillin production (%)
0	100	100	0	100	100
0·01	100	92	0·01	69	24
0·02	91	54	0·02	17·9	0
0·04	62·5	33	—	—	—
0·08	42	0	—	—	—

Malonate

Concentrations of sodium malonate up to 0·2 M had no effect on respiration or on penicillin production; higher concentrations interfered with the assay of penicillin. The absence of inhibition of respiration by malonate is probably because the pH value at which these experiments were carried out was too high to allow a sufficient concentration of undissociated acid. Turner & Hanly (1947) reported that 0·05 M-malonate inhibited oxygen uptake by carrot root slices at pH 5·0 and less, but stimulated respiration at pH 6·0 and greater. Experiments with *Penicillium chrysogenum* were not carried out at pH values lower than 6·0 because of the rapid fall in the rate of penicillin production as the pH was lowered.

Azide

Sodium azide did not inhibit penicillin production to any markedly greater extent than total respiration; results of a typical experiment are shown in Table 2. With 0·0001 and 0·0002 M-azide, the figures for penicillin production are somewhat lower than for oxygen uptake but the effect is not marked and

Table 2. *Effect of sodium azide on penicillin production and oxygen uptake by* Penicillium chrysogenum.

Rates of penicillin production and oxygen uptake as percentage of that obtained without azide.

Sodium azide (M)	Rate of O_2 uptake (%)	Rate of penicillin production (%)
0	100	100
0·0001	92	77·5
0·0002	57	36
0·0005	20	20
0·001	15	8·1

hardly comparable with the effect of fluoride or bisulphite. However, azide is known to be a powerful inhibitor of respiration, and any specific effect on penicillin production might well be masked.

2:4-*Dinitrophenol*

Penicillin production was very much more sensitive to 2:4-dinitrophenol than was respiration (Fig. 2). A concentration of 0·0004 M caused almost no inhibition of respiration but penicillin production was depressed to *c.* 50 % of maximum. With 0·0006 M, penicillin production was almost completely inhibited yet respiration was still *c.* 60 % of maximum.

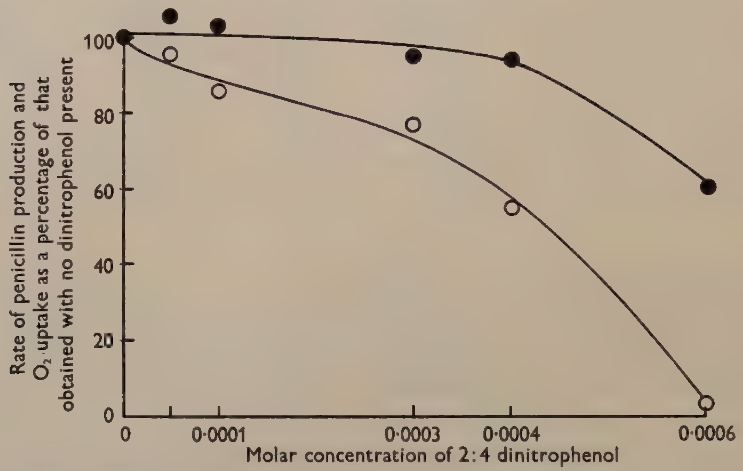

Fig. 2. Effect of 2:4-dinitrophenol on penicillin production (○——○) and oxygen uptake (●——●) by *P. chrysogenum*.

Cyanide

Penicillin production was extremely sensitive to cyanide (Figs. 3 and 4); 0·000002 M-KCN, which had no significant effect on oxygen uptake, inhibited penicillin production to *c.* 25 % of the maximum. A 50-fold increase in cyanide concentration (i.e. to 0·0001 M) was required to bring about a similar degree of inhibition of respiration. Penicillin production and respiration both appeared to contain some proportion or component which was relatively cyanide stable (Fig. 3). A concentration of 0·001 M-KCN had little greater effect on penicillin production than did 0·000002 M, the latter being sufficient to depress penicillin production to *c.* 25 % of maximum, and a concentration of 0·005 M-KCN (i.e. an increase of over 2000-fold) was required to inhibit penicillin production completely. Similarly, although a concentration of 0·0001 M-KCN inhibited respiration to *c.* 25 % of maximum a concentration of 0·005 M-KCN (a 50-fold increase) was required to inhibit respiration completely. The results in Fig. 3 also show that when poisoned with cyanide (e.g. with 0·0001 M-KCN) the cyanide-stable proportion allows both penicillin production and respiration to proceed at the same reduced rate, i.e. *c.* 25 % of maximum.

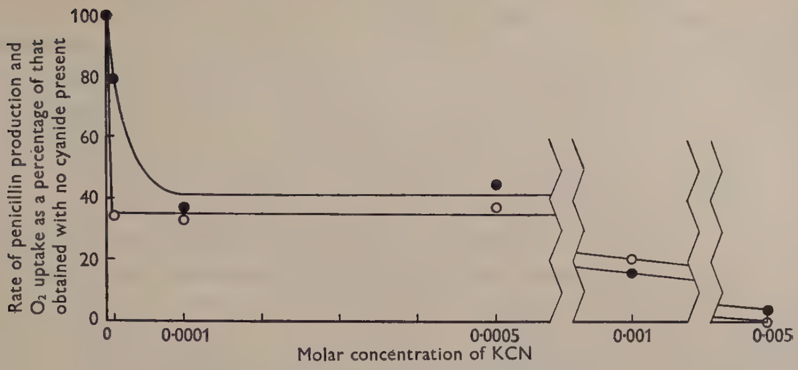

Fig. 3. Effect of cyanide on penicillin production (○——○) and oxygen uptake (●——●) by *P. chrysogenum.*

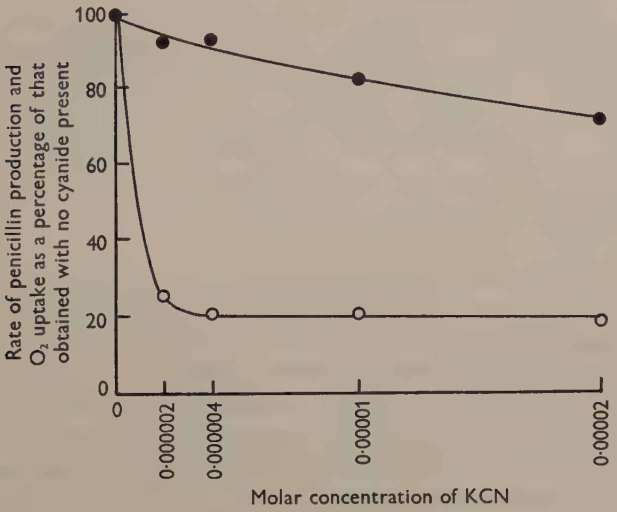

Fig. 4. Effect of relatively low concentrations of cyanide on penicillin production (○——○) and oxygen uptake (●——●) by *P. chrysogenum.*

DISCUSSION

The results with phenol and mercuric chloride show that penicillin production is not always depressed preferentially, compared with some other cell process such as respiration, regardless of the inhibiting agent. The results with phenol were somewhat complicated by the slight stimulation of respiration at low concentrations, an effect similar to that reported by Hugo & Street (1952) with *Escherichia coli.*

The results with fluoride and bisulphite suggest that these substances interfere either directly with the mechanism of penicillin production or by preventing some other processes which furnish intermediates in the formation of penicillin. The results with 2:4-dinitrophenol were essentially similar to those obtained in experiments on other synthetic processes such as the synthesis of proteins and enzymes (Clifton, 1946; Hotchkiss, 1947; Frantz, Zamecnik, Reese & Stephenson, 1948; Fowler, 1951); concentrations which had little effect on total oxygen uptake markedly inhibited penicillin synthesis. This suggests that phosphate bond energy is utilized in the synthesis of penicillin, as in the other synthetic processes previously mentioned, since 2:4-dinitrophenol is known to uncouple respiration from phosphorylation (Loomis & Lipmann, 1948).

The results with cyanide show that a cyanide-sensitive system is involved for maximum oxygen uptake and penicillin production but that an alternative, and relatively cyanide-insensitive, system allows oxygen uptake and penicillin production to proceed at *c.* 25% of the maximum rate. The inhibition of penicillin production by cyanide would appear to be a direct effect because penicillin production and respiration were not equally sensitive to cyanide. A concentration of 0·000002 M-KCN decreased penicillin production to only *c.* 25% of the maximum rate, i.e. the cyanide-sensitive system was completely blocked yet the rate of oxygen uptake was not significantly affected. A concentration of 0·0001 M was required to decrease the respiration to *c.* 25% of maximum. This does not, however, rule out the possibility that the cyanide-sensitive systems in penicillin production and respiration are the same, since those enzyme molecules concerned with penicillin synthesis might conceivably be more accessible or sensitive to cyanide. In either case, the results indicate that a cyanide-sensitive enzyme is involved in the biosynthesis of the penicillin molecule.

There is no evidence as to the nature of such an enzyme. Inhibition with cyanide suggests an iron- or copper-containing enzyme. It may be significant that iron is essential for penicillin production as distinct from growth (Jarvis & Johnson, 1950); approximately 20 times as much iron being required for maximum penicillin production as for maximum growth only.

Despite the differences in the relative sensitivity to cyanide, the effects on respiration and penicillin production have something in common. When the respective cyanide-sensitive systems are completely blocked (i.e. with 0·0001 M-KCN) both respiration and penicillin production proceed at the same reduced rate of *c.* 25% of maximum. This suggests that the cyanide-stable mechanism may be common to both penicillin production and respiration, i.e. be concerned with hydrogen transfer in both processes. As a working hypothesis, therefore, it is suggested that one step in the biosynthesis of penicillin involves an oxidation and that a cyanide-sensitive enzyme is essential in order that this step should proceed at the maximum rate. Further, a cyanide-sensitive system seems to be essential for the maximum rate of respiration and this may or may not be identical with that involved in penicillin production. It is further suggested that an alternative enzyme system, relatively insensitive to cyanide, is present which allows hydrogen transfer to proceed at a limited rate, so that both respiration and penicillin production proceed at *c.* 25% of the maximum rate. The oxygen uptake involved in such an oxidative step in penicillin synthesis might account for only a very small proportion of the total oxygen uptake and might not be noticed when penicillin synthesis was blocked by low concentrations of cyanide which had little effect on the total oxygen uptake.

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The Morphology of *Nocardia opaca* Waksman & Henrici (*Proactinomyces opacus* Jensen) when Grown on Hydrocarbons, Vegetable Oils, Fatty Acids and Related Substances

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SUMMARY: *Nocardia opaca* Waksman & Henrici can use certain long chain saturated aliphatic hydrocarbons and many vegetable oils as sole carbon and energy source in presence of simple mineral salts. Long chain saturated aliphatic acids (e.g. myristic, palmitic and stearic) can similarly serve.

The same general morphological development is obtained on all these compounds but the initial mycelial phase is more pronounced on oily substrates (particularly hydrocarbons) than on water soluble or insoluble solid substances. Granules are demonstrable inside the cells. These granules are more intensely stained with fat and polysaccharide reagents than the rest of the cell. Well-defined electron dense bodies are also observed which behave similarly to the 'mitochondria' described by Mudd, Winterscheid, DeLamater & Henderson (1951) in *Mycobacterium thamnopheos* under intense electron bombardment.

Webley & de Kock (1952) showed, by means of the Warburg technique, that the addition of certain long chain saturated aliphatic hydrocarbons, alcohols and acids to washed suspensions of *Nocardia opaca* grown on a glucose containing medium enhanced oxygen uptake. In the present paper a study has been made of the growth and morphology of this organism with these substances as sole carbon and energy sources. The work was extended to include vegetable oils, some waxes and other related substances.

METHODS

Organism. Nocardia opaca strain T_{16} was used throughout (Webley & de Kock, 1952).

Growth experiments were carried out in 2 oz. screw-capped bottles containing 5 ml. medium. A mineral medium of the following composition was used throughout (%, w/v): $NaNO_3$, 0·2; NaCl, 0·02; $MgSO_4.7H_2O$, 0·02; $FeCl_3$, trace. The pH value of the solution was adjusted to 7·2.

Carbon compounds used. For the oily substances 0·2 ml./bottle of each was used. For solid water-insoluble substances, a few flakes of each substance were added to the salt solution in the 2 oz. bottles before autoclaving. Fatty acids were used as sodium or potassium salts. Thus the only carbon source, apart from atmospheric CO_2, involved in the growth experiments, was the carbon compound added to the salt solution. Whenever possible highly purified chemicals were used.

Inoculum. One or two drops of a thin suspension of *Nocardia opaca* in distilled water was used to inoculate each culture bottle. This suspension was prepared by adding a sufficient amount of organism, obtained from the surface of solid medium, to 5 ml. sterile distilled water in a 2 oz. screw-capped bottle containing a few glass beads. For this purpose the organism was grown on the medium used by Webley & de Kock (1952) for 2 days at 25°. As it is difficult to standardize a suspension of a pleomorphic organism by counting methods the final suspension was made so as to give a slight turbidity visible to the naked eye after shaking by hand for $\frac{1}{2}$–1 min. with the glass beads. After inoculation the bottles were incubated at 23–25° and examined daily. Owing to the physical nature of most of the substrates and the mode of growth of the organism on them it was not possible to assess the amount of growth by conventional methods.

Examination of growth. Examination by microscope was periodically carried out by the ordinary hanging-drop technique; at the same time heat-fixed smears were made for the following staining reactions: (*a*) Gram's method (Hucker's modification); (*b*) acid-fast stain (Gordon & Smith, 1953); (*c*) fat stain (Burdon, 1946); (*d*) polysaccharide stain of Hotchkiss as described by Glick (1949). In method (*d*) the ethanolic periodic acid 'A' solution was used and the counter-stain omitted. The periodic acid solution was allowed to act for 1 hr., while the Feulgen reagent (prepared by reduction with SO_2) was subsequently used for 20 min. The rest of the procedure was as described by Glick (1949).

Preparation of specimens for electron microscope examination. Small drops, obtained by dipping the end of an ordinary platinum inoculation wire down to the oil/aqueous interface in the case of the oils (i.e. the site of growth of the organism), were placed on copper electron microscope specimen grids which had been covered with a thin formvar film. The specimens were fixed in formalin vapour and then dried in a desiccator overnight before mounting in the microscope.

RESULTS

Growth of Nocardia opaca *on saturated aliphatic hydrocarbons*

The organism grew rapidly (2–3 days) on the long chain hydrocarbons *n*-dodecane (C_{12}), *n*-tetradecane (C_{14}) and *n*-hexadecane (C_{16}) and slightly less rapidly on the solid *n*-octadecane. Growth on lower members of the series (heptane, octane, nonane) was poor in comparison. In the case of the C_{12}, C_{14}, C_{16} hydrocarbons, which were oils at the temperature used, growth took place at the oil/water interface. *n*-Octadecane is a solid below 28°, and the organism grew first at the solid surface and then settled to the bottom of the tube, although flakes of hydrocarbon might remain floating at the surface. At 30–31°, when *n*-octadecane is liquid, growth took place as for the C_{12}, C_{14} and C_{16} hydrocarbons, the cells sticking firmly to the oil and only depositing after most of the hydrocarbon had been used.

The initial mycelial phase was well developed on the oils (Pl. 1, figs. 1, 2). The cells were acid-fast and had deeper staining Gram-positive granules inside the slender mycelium (Pl. 1, fig. 1). These granules also stained more deeply than the rest of the cell with fat or polysaccharide stains (Pl. 1, figs. 3, 4). Under the electron microscope well-defined granules were seen inside the mycelium (Pl. 1, fig. 2). In short elements these granules were usually situated at the poles of the cell (Pl. 1, fig. 5). Increasing the intensity of the electron beam produced 'vacuolation' of the granules (Pl. 1, fig. 6) in an identical manner to that obtained by Mudd *et al.* (1951) for *Mycobacterium thamnopheos* and more recently by Brieger, Cosslett & Glauert (1954) for strains of *M. avium.*

Growth of Nocardia opaca *on saturated aliphatic alcohols*

No growth, or very little, was obtained with the following alcohols: butyl, amyl, hexyl, heptyl, octyl, decyl, undecyl and dodecyl. It was previously found (Webley & de Kock, 1952) that butyl, amyl, hexyl and heptyl alcohols were inhibitory to the respiration of washed suspensions of the organism. On the other hand, enhanced oxygen uptake was obtained in the presence of decyl and dodecyl alcohols. Growth, however, took place on cetyl alcohol (C_{16}) and on stearyl alcohol (C_{18}). On the latter compound the initial mycelial phase was short lived and the mycelium quickly fragmented into the short element phase.

Growth of Nocardia opaca *on saturated aliphatic fatty acids*

Lower fatty acids (C_2–C_7). Good growth takes place on acetate, *n*-butyrate, *iso*-butyrate, *n*-hexanoate and *n*-heptanoate at 0·5 % (w/v). The development of the initial mycelial phase was not so well marked as with the hydrocarbons; fragmentation tended to take place more quickly. The usual granules which stained with the polysaccharide and fat stains were observed. Higher homologues in this series of acids under similar conditions did not support growth.

Higher fatty acids (C_8–C_{20}). By lowering the concentration to 0·001 M growth was obtained on the following straight chain fatty acids: caprylic, capric, myristic, palmitic, stearic and arachidic. The initial mycelial development of the organism with these compounds was short lived (shorter than with the lower fatty acids—see above), and it fragmented into the short element phase (Pl. 1, fig. 7) after 2–3 days. Most of the cells deposited at the bottom of the culture bottles. The growth, which took place at the surface of the culture medium, was composed of highly refractile cells possessing a marked tendency to creep up the sides of the bottle on shaking. Very few cells remained suspended in the liquid medium itself when these fatty acids were used. The cells were acid-fast and Gram-positive, with internal granules which stained in a similar manner to those obtained in the growth on hydrocarbons.

Growth of Nocardia opaca *on vegetable oils and waxes*

Vegetable oils. Very good growth (2–3 days) was obtained on the following vegetable oils: olive, almond, castor, rape seed, maize, soya bean, safflower seed, tea seed, ground-nut, cotton seed and cocoa-nut. Growth took place at the oil/water interface in the same manner as that observed with the hydrocarbon oils. The cells and internal granules stained in a similar manner to that described for growth on the hydrocarbons. Webley (1953) showed that a well-developed mycelial stage was initially produced by *Nocardia salmonicolor* when growing on vegetable oils.

Waxes, etc. The following waxy substances were tried: beeswax, sandal leaf wax and synthetic palmitone. Beeswax was the only one of these substances on which the organism grew. On beeswax the cells did not attach themselves so firmly to this solid in the manner observed for oily substances (see also comparative behaviour in *n*-octadecane when in the oil and solid phase). Fragmentation quickly took place into the short element phase and most of the cells settled to the bottom of the culture bottle.

Growth of Nocardia opaca *on various surface-active and related substances*

Many surface-active substances possess paraffinic chains in their structures (Glassman, 1948). Bearing this in mind and also the well-known effect of 'Tween 80' on the growth of *Mycobacterium tuberculosis* (Dubos & Middlebrook, 1948) it was considered worthwhile to try these compounds as sole carbon source for *Nocardia opaca.* It was found that while cationic and anionic surface active compounds containing a paraffinic chain did not serve as sole C source for *N. opaca,* Tween 80, on the other hand, at 0·1 % supported growth of the organism; the sodium salt of cholic acid also gave good growth at a similar concentration. Little growth at the liquid/air surface was obtained with Tween 80 or sodium cholate; the cells quickly settled to the bottom of the culture bottle and were in the short element phase. A similar result was obtained with 0·1 % bile salt as sole carbon source. *N. opaca* grew with cholesterol as sole carbon source; Turfitt (1944) showed that species of *Proactinomyces* used cholesterol as sole carbon source.

DISCUSSION

The ability of the partially acid-fast nocardias to grow on liquid or solid paraffins is well known (Jensen, 1931; Erikson, 1949). Erikson (1949) has also shown that the proportion of this type of organism among soil nocardias is quite considerable. Thus out of 300 soil strains she studied 31 % were partially acid-fast. The finding that *Nocardia opaca* can grow rapidly on vegetable oils and also utilize long chain fatty acids as carbon source indicates that this group of organisms may be of importance in the breakdown of lipid material which takes place in soil.

The reason for the more pronounced development and persistence of the initial mycelial phase on oils (particularly hydrocarbons) may be connected with the rather different physical factors operating at the oil/water interface as compared with those in aqueous solutions and in solid + water systems.

The presence of well-defined granules inside the cells was reported and studied in detail by McClung (1950) who grew the organisms mainly in medium containing glycerol. In the present investigation granules were also observed in the cells of *Nocardia opaca.* They stained more intensely than the rest of the cell with fat and with polysaccharide stains. Under the electron microscope well-defined dense bodies were seen (Pl. 1, figs. 2, 5) which behaved in a similar manner to the 'mitochondria' in *Mycobacterium thamnopheos* (Mudd *et al.* 1951) under intense electron bombardment. Whether these bodies are identical with the granules observed by staining reactions (Pl. 1, figs. 3, 4) is not known with certainty. Comparison of their positions in stained preparations and electron micrographs support the view that they are.

The refractility of the cells and internal granules of *Nocardia opaca*, as of other fat-utilizing partially acid-fast species of *Nocardia*, is a property that allies this class of organism more closely with the mycobacteria than with the other group of organisms still retained in the genus *Nocardia*—viz. the non-acid-fast, non-fat utilizing, carbohydrate fermenting species that have only weakly refractile cells with poorly visible granules, e.g. *N. flava*, *N. citrea*, etc. (Erikson, 1954).

I am grateful to Mrs D. Oxford and Dr G. K. Fraser for their continued interest throughout the work. I am indebted to Mr Gard of the University of Aberdeen for taking the electron micrographs and to Miss Irene Taylor for the photomicrographs. I also wish to thank Mr Rae of the United Africa Co. Ltd. for sending me samples of refined and deodorized vegetable oils, Dr S. G. Knight for samples of surface-active agents not obtainable in this country, Dr M. Webb for the purified sample of cholic acid, Messrs Hess Products Ltd., Littleborough, for the long chain fatty acids and to others for gifts of hydrocarbons of high purity. The author is also grateful to Dr A. C. Chibnall, F.R.S., for the gift of sandal leaf wax, and synthetic palmitone.

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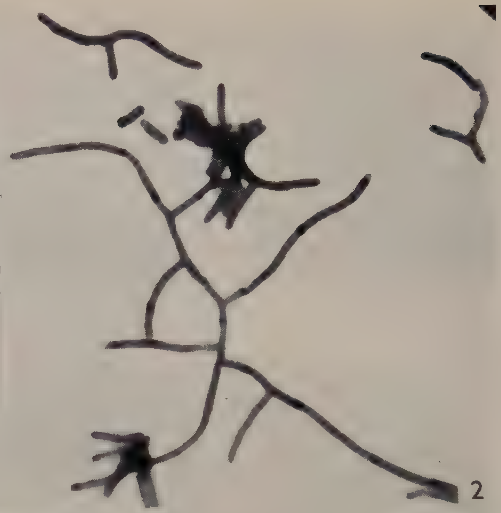

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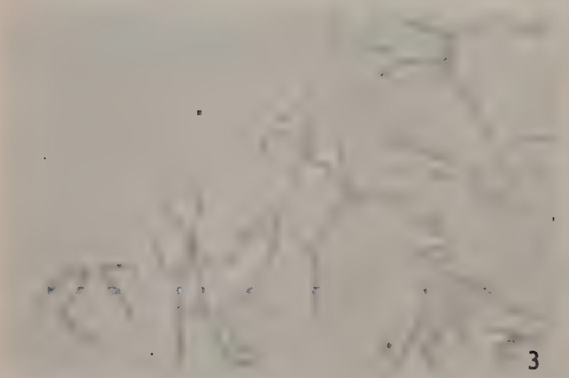

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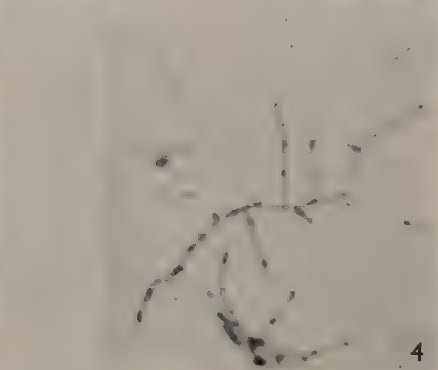

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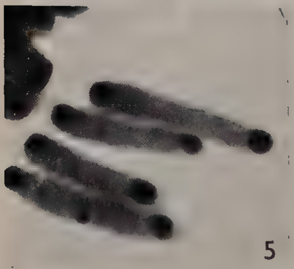

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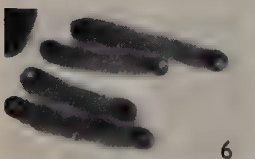

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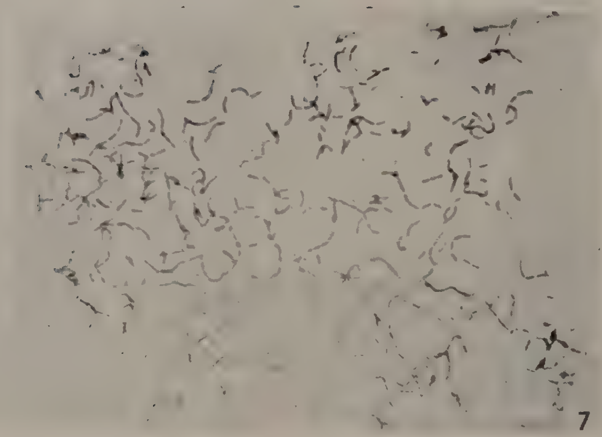

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EXPLANATION OF PLATE

Fig. 1. *Nocardia opaca* grown for 4 days on *n*-dodecane and mineral salts. Gram stain, × 960.

Fig. 2. *N. opaca* grown for 3 days on *n*-dodecane and mineral salts. Electron micrograph, × 3700.

Fig. 3. *N. opaca* grown for 4 days on *n*-dodecane and mineral salts. Fat stain, × 1920.

Fig. 4. *N. opaca* grown for 2 days on *n*-dodecane and mineral salts. Polysaccharide stain, × 1920.

Fig. 5. *N. opaca* grown for 2 days on *n*-dodecane and mineral salts. Electron micrograph, × 12,500.

Fig. 6. As above after intense electron bombardment. Electron micrograph, × 10,000.

Fig. 7. *N. opaca* grown for 3 days on 0·001 M-Na palmitate and mineral salts. Gram stain, × 960.

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Induced Biosynthesis of Lysine Decarboxylase in *Bacterium cadaveris*

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SUMMARY: Experiments have been carried out on the kinetics of induced synthesis of lysine decarboxylase in washed suspensions of *Bacterium cadaveris*. The enzyme is produced when glucose and lysine are present simultaneously. Lysine analogues tested as inducers were inactive. In anaerobic synthesis the rate of formation of new enzyme is directly proportional to the degree of saturation of the enzyme by its substrate; in aerobic synthesis a maximal rate is found even when the enzyme is almost totally unsaturated. The pH curve for enzyme synthesis corresponds closely with that for enzyme activity. Adapted cells undergo de-adaptation when incubated with glucose in the absence of lysine; this is due to the effect of cadaverine on the co-enzyme. The bearing of these results on current theories of enzyme induction is discussed.

In experiments previously reported, induced enzyme synthesis in yeast was examined from the standpoint of the 'mass action theory of enzyme adaptation' (Mandelstam & Yudkin, 1952). According to this theory, the inducible enzyme is in equilibrium with a precursor; addition of the substrate, or other inducer, upsets the equilibrium which is then restored by further enzyme synthesis (Yudkin, 1938). The postulates are simple and the theory can be readily expressed in quantitative terms (Mandelstam, 1952). It was found that when cells were adapted to galactose and maltose, the results were in close agreement with the qualitative and quantitative predictions made.

Although the theory thus appears to hold for these enzymes in yeast it is in conflict with observations made on other systems. For instance, Monod, Cohen-Bazire & Cohn (1951) showed that for β-galactosidase synthesis in *Escherichia coli* there was no correlation between inductive activity and affinity for the enzyme. Similarly, penicillinase formation in *Bacillus cereus* can be induced by minute amounts of penicillin and the process continues at an undiminished rate in the absence of any detectable free substrate (Pollock, 1950). It seems that in this case, and in that of β-galactosidase, the inducer acts by combining with something other than the enzyme, and that the complex thus formed catalyses the synthesis of the enzyme. This can be schematically represented as follows:

$$\begin{array}{c} \mathrm{I}+\mathrm{R} \\ \upharpoonleft\downharpoonright \\ \mathrm{IR} \\ \mathrm{B} \dashrightarrow \mathrm{E} \end{array}$$

B is a precursor or pool of precursors, E is the enzyme, I the inducer, R the receptor and IR is the complex which catalyses the synthesis of E. The broken arrow indicates that a number of reactions may be involved. (In the case of penicillin the reaction with the receptor seems to be irreversible.)

The 'catalyst' theory may be compared with the 'mass action' theory:

$$B \rightleftharpoons E + I \rightleftharpoons EI.$$

Addition of inducer I upsets the equilibrium between the enzyme E and the pool of precursors B, and the system adjusts itself by producing more enzyme. It follows that the initial rate at which enzyme is synthesized will be proportional to the degree of saturation of E. On the other hand, if the 'catalyst' theory holds, the rate of synthesis will depend on the degree of saturation of R. A kinetic study of enzyme production might be expected to provide evidence for differentiating between the two mechanisms, unless R and E happened to to have the same affinity for the inducer.

The system chosen for investigation was lysine decarboxylase in *Bacterium cadaveris*, because only one enzyme is involved; and because the cells do not depend on the inducing substrate for their supply of energy.

MATERIALS AND METHODS

The organism used was *Bacterium cadaveris* (NCTC 6578). The growth medium contained: KH_2PO_4, 4·5 g.; NH_4Cl, 0·5 g.; $(NH_4)_2SO_4$, 0·5 g.; $MgSO_4$, 20 mg.; $Fe(NH_4)_2(SO_4)_2$, 15·6 mg.; glucose, 20 g.; sodium lactate, 20 g.; and water to 1 l. The pH value was adjusted to 7·5 and the organisms grown with shaking at 35° for 18 hr., by which time growth was approaching the end of the logarithmic phase and the culture contained about 0·9–1·0 mg. dry weight bacteria/ml.

Enzyme induction. Unless otherwise stated, enzyme induction and assay, and the washing of bacteria were carried out in 0·05 M-phthalate buffer at pH 5·5. For induction, the cells were incubated at 30° with 1 % glucose and lysine of varying concentrations (see below). The bacterial density was 0·15 mg. dry weight bacteria/ml. Five ml. samples were removed at intervals and added to 1 ml. of 10^{-3} M-sodium azide. This concentration of azide was sufficient to prevent any further induction but had no inhibitory effect on formed enzyme. The cells were then washed and resuspended in 5 ml. of buffer solution and 2·5 ml. taken for assay. Activity was measured anaerobically in Warburg manometers at 30° using 0·5 ml. of 0·067 M-lysine in the side-arms. For estimation of the apo-enzyme, the cells were shaken with a drop of toluene and left at room temperature for 20 min.; the toluene was then removed by a slow stream of air and the activity measured in the presence of pyridoxal phosphate (12·5 μg./ml.). The toluene treatment was found to be essential because pyridoxal phosphate fails to penetrate untreated cells. Carbon dioxide production was somewhat erratic during the first 5 min., but steady readings were obtained for the next 20 min. The CO_2 evolved under these conditions was proportional to the enzyme present. Enzyme activity is expressed in arbitrary units (μl. CO_2/20 min.). Bacterial concentrations were determined by opacity measurements using a Spekker absorptiometer with filter 508, and a calibration curve of opacity against dry weight/ml.

Special reagents. Cadaverine was prepared by the action of hydrazoic acid on pimelic acid (Oesterlin, 1932); the picrate was isolated and recrystallized from hot water, and the dihydrochloride prepared from it in the normal way. L-ϵ-*N*-benzoyl-lysine and L-ϵ-*N*-acetyllysine were prepared from L-lysine by the methods of Sanger (1946) and Neuberger & Sanger (1943). DL-ϵ-hydroxy-α-aminocaproic acid was synthesized from dihydropyran (Gaudry, 1948). α-ϵ-Diaminoheptanoic acid was prepared from *O*-methylcyclohexanone via ϵ-*N*-benzoyl-aminoheptanoic acid (method of McLaren & Knight, 1951), and subsequent chlorination, amination and hydrolysis carried out by the method of Galat (1947).

RESULTS

Stability of lysine decarboxylase

To begin with, it was necessary to examine the stability of lysine decarboxylase in view of Gale's statement (1940) that the amino acid decarboxylases may be markedly unstable even at 30°. It was found that there was no detectable loss of activity even after 3 hr., when cells were incubated in buffer at 30°. The fall in CO_2 production commonly observed after the first half hour of manometric measurement was due to exhaustion of substrate. The initial rate of decarboxylation could be restored by resuspending the cells in fresh buffer or by adding more lysine from a second side arm.

Conditions for induction of lysine decarboxylase in washed suspensions

When washed cells were incubated with glucose and lysine (0·011 M) at 30° a linear rate of synthesis of lysine decarboxylase was found (Fig. 1). An exponential rate of increase was not observed under any of the conditions of induction tested. In aerobic induction there was a lag period of about 10 min. (often much less) before the linear phase of enzyme production began; anaerobically there was no lag. L-Lysine and DL-lysine of equivalent concentration were equally effective, the D-isomer having no apparent effect either as an inducer or as an inhibitor of induction. During induction there was no increase in cell mass as measured by opacity, nor was there any detectable alteration in the pH. For adaptation to occur at all, it was necessary for glucose and lysine to be present simultaneously. No increase in activity was caused by treatment with glucose, either alone or in conjunction with ammonium salts, magnesium or ATP. When NH_4Cl was added together with lysine and glucose the rate of increase of activity was not affected, but the final level was about 10 % higher.

The effect of pH *on the rate of formation of enzyme*

Washed cells were incubated aerobically with glucose and lysine (0·11 M) in buffer solutions (phthalate was used for pH 4·5–5·8; phosphate at higher pH values, with overlap at pH 5·8). Samples were taken at intervals during the first 90 min. of incubation and added to the azide solution. The cells were washed and activity determined at pH 5·5 in the usual way. The rate of increase of activity was measured by the slope of the line. Owing to the practical difficulty of doing more than three curves simultaneously, the following procedure was adopted to obtain a composite curve of adaptation against pH value. The rate at pH 5·5 was arbitrarily taken as 100 and the rates at two other pH values expressed as fractions of this value. The final curve is shown in Fig. 2, together with the curve for enzyme activity. The latter was obtained by suspending washed cells in the appropriate buffer solutions and measuring CO_2 production manometrically. Readings were corrected for CO_2 retention.

It will be seen that the curve for enzyme synthesis follows very closely that for enzyme activity, and both are optimal at pH 5·3–5·6. A similar curve was found when enzyme induction was carried out anaerobically. That this pH effect was an effect on the synthesis of the apo-enzyme was shown by estimating the activity in toluene-treated cells in the presence of pyridoxal phosphate. Higher absolute values were obtained but the relationship between the rates was unaffected.

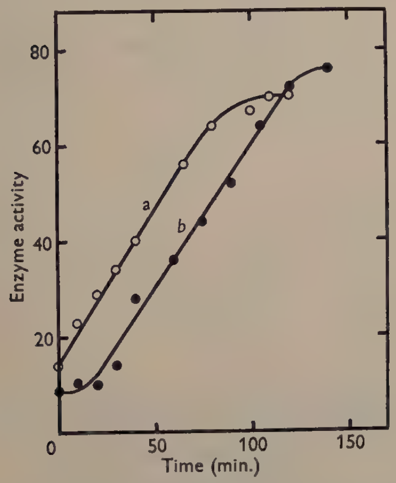


Fig. 1

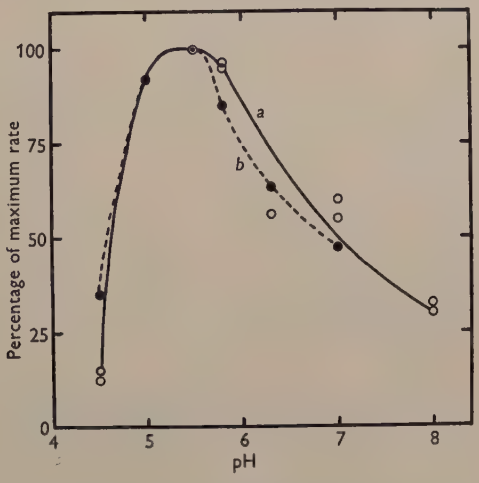


Fig. 2

Fig. 1. Induction of lysine decarboxylase activity in washed cell suspensions by incubation with lysine (0·011 M) and glucose (1 %): (*a*) anaerobic induction; (*b*) aerobic induction.

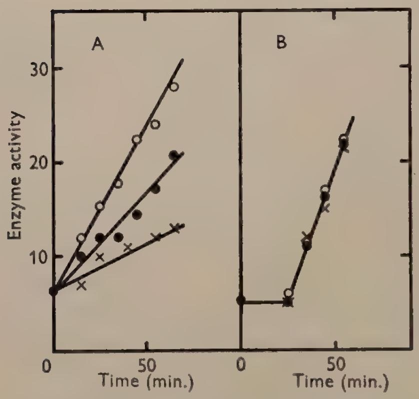
Fig. 2. Effect of pH on the rate of aerobic synthesis of lysine decarboxylase (*a*) compared with the effect on the activity of the enzyme itself (*b*).

The effect of concentration of lysine on the rate of formation of enzyme

Anaerobic induction. When cells were incubated with lysine and glucose in an atmosphere of nitrogen the rate of synthesis of the enzyme increased with the concentration of lysine (Fig. 3A) and was maximal at 0·022 M. To plot a composite curve, the slope of the line at 0·022 M in each experiment was taken as 100 and the slopes at two other concentrations expressed as percentage fractions. The results of this experiment are shown as the points in Fig. 4. When plotted by the method of Dixon (1953) the points lie about a straight line. The value for 50 % maximal rate of enzyme formation is 0·0013 M.

For comparison the Michaelis constant for lysine decarboxylase was determined anaerobically using intact cells and a series of seven lysine concentrations over the range 0–0·022 M. The value found for K_m was 0·0018 M. With intact cells in the presence of 1 % glucose $K_m = 0{\cdot}0019–0{\cdot}0020$ M, while with acetone-dried preparations $K_m = 0{\cdot}0017$ M. It is clear that glucose does not significantly affect the penetration of lysine into the cell. It is apparent that the rate of enzyme production is closely correlated with the degree of saturation by the substrate. This relationship was found to hold for the apo-enzyme as well as the activity of intact cells.

Aerobic induction. The effect of the concentration of lysine on the rate of enzyme formation was examined over the same range as before, but with the induction carried out aerobically. The results were markedly different (cf. Fig. 3 A, B). The rate of synthesis was constant over the range tested, and the concentration of lysine could be further reduced to 10^{-4} M without significantly affecting the initial rate.



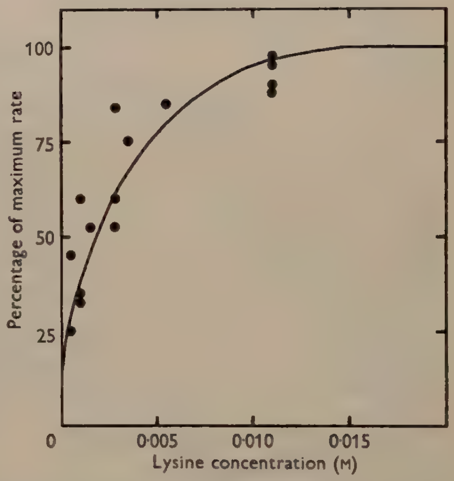


Fig. 3. Effect of lysine concentration on the rate of synthesis of enzyme anaerobically (A) and aerobically (B). ○—○, 0·022 M; ●—●, 0·0014 M; ×—×, 0·0007 M.

Fig. 4. Effect of lysine concentration on the rate of anaerobic enzyme synthesis compared with the effect of lysine concentration on enzyme activity. The points give the rates of synthesis as a percentage of the rate at 0·022 M lysine; the continuous curve is the saturation curve for lysine decarboxylase.

It thus appears that when the enzyme is induced aerobically its rate of appearance is maximal even at low concentrations of the inducer and is largely independent of the degree of saturation of the enzyme. The situation is similar to that found with β-galactosidase, and is consistent with a 'catalyst' mechanism. On the other hand, in anaerobic induction, the rate follows the saturation curve of the enzyme—a result anticipated on the basis of a 'mass action' mechanism. This apparent discrepancy might be explained in a number of ways:

(1) The enzyme is produced in both cases by a 'mass action' process; the

aerobic induction appears to be discrepant because the cells concentrate lysine so that the enzyme is saturated even when the external concentration is low.

(2) The enzyme is produced 'catalytically' in both cases, but under anaerobic conditions the inducer is becoming rapidly exhausted.

(3) The enzyme is produced 'catalytically'; the anaerobic induction appears to be discrepant because the enzyme formed under these conditions is unstable but is stabilized by the substrate so that the amount of enzyme measured reflects the degree of saturation.

Experiments were then carried out to test these possibilities.

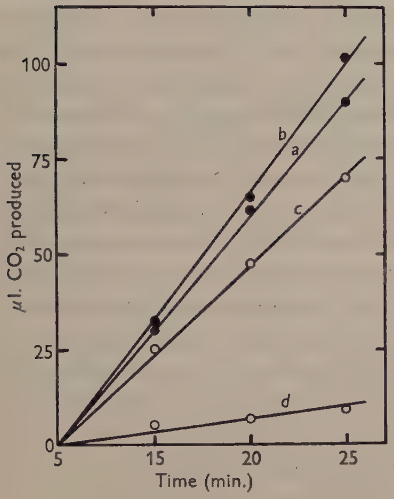


Fig. 5

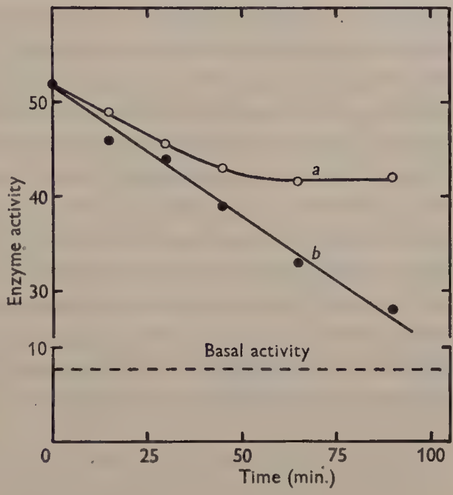


Fig. 6

Fig. 5. Effect of glucose on penetration of lysine into the cell: (*a*) anaerobic decarboxylation of lysine; (*b*) anaerobic decarboxylation of lysine in presence of 1 % glucose; (*c*) aerobic decarboxylation of lysine; (*d*) aerobic decarboxylation of lysine in presence of 1 % glucose. Values shown are corrected for 'blanks'. In presence of glucose, CO_2 production by 'blanks' anaerobically and aerobically, was 39 μl. and −10 μl./30 min. respectively.

Fig. 6. Loss of adaptive activity produced by incubating cells with glucose in the absence of lysine: (*a*) anaerobic incubation; (*b*) aerobic incubation. Activity measured on intact cells.

(1) *The effect of glucose on penetration of lysine into the cell*

To determine whether lysine was being concentrated in the cells under the experimental conditions for induction, a suspension of washed cells in buffer was placed in Warburg flasks with 0·5 ml. lysine solution or 0·5 ml. H_2O (blanks) in the side-arms. A similar series of flasks contained glucose (final concentration 1 %). Production of CO_2 was measured aerobically and anaerobically, the first 5 min. being neglected. In the absence of glucose the rate of decarboxylation was slightly lower aerobically. In the presence of glucose, the aerobic decarboxylation was greatly retarded, while the anaerobic decarboxylation was, if anything, slightly increased (Fig. 5). A similar aerobic retardation due to glucose was found at all concentrations of lysine tested over the range of 0·0007 M–0·011 M. This experiment makes the first possibility considered above untenable. It is apparent that under the conditions of aerobic induction, the lysine, far from being concentrated in the cell, actually fails to penetrate freely. A similar observation on the effect of glucose on the penetration of lysine was made by Gale (1947).

(2) *Utilization of lysine during induction*

The second possible explanation for the difference between aerobic and anaerobic induction was that, in the latter, the lysine was becoming rapidly exhausted. This possibility was tested as follows. Induction at two different concentrations of lysine was carried out in the usual way. At 60 min., measured samples were taken for lysine analysis and heated in boiling water for 15 min. to destroy enzyme. The cells were removed by centrifuging, and the residual lysine in the supernatant fluid estimated manometrically using chloramine T (Cohen, 1940). As this method tends to give high values, a calibration curve had to be made using known concentrations of lysine. The results obtained in two experiments are shown in Table 1. In the case of the more concentrated lysine solutions, the amount destroyed during induction was not significant, and even in the more dilute solutions 70–80 % of the amount added was intact at the end of an hour. There is, therefore, no reason for assuming that the difference between aerobic and anaerobic induction is due to a rapid utilization of lysine under anaerobic conditions.

Table 1. *Destruction of lysine during initial stages of anaerobic and aerobic induction of lysine decarboxylase*

Conditions of induction	Initial lysine concn. (M)	Lysine concn. after 1 hr. (M)	% lysine intact after 1 hr.
Anaerobic	0·0111	0·0107	96·5
Aerobic	0·0111	0·0112	100
Anaerobic	0·00069	0·00049	71
Aerobic	0·00069	0·00055	80

(3) *Stability of lysine decarboxylase*

The following experiments were done to determine whether induced lysine decarboxylase was inherently unstable during induction and was stabilized by the substrate. The question of stability was examined from two points of view: the behaviour of the intact cells and the stability of the apo-enzyme.

Cells were incubated anaerobically with glucose and lysine. After 100 min. they were centrifuged and resuspended, without washing, in the same volume of buffer containing glucose but no lysine. The suspension was divided into portions, one of which was incubated aerobically and the other in an atmosphere of nitrogen. Samples were taken periodically and the activity of the intact cells measured. In both suspensions there was a loss of activity which was more rapid in air (Fig. 6). It was also found that whether the induction had taken place aerobically or anaerobically there was no difference in the subsequent behaviour. Addition of lysine (0·01 M) to the system reversed the loss and, if added at the start, prevented it altogether.

Next, lysine-adapted cells were washed twice before being resuspended in buffer and glucose. This retarded the loss of activity and occasionally prevented it completely. However, loss of activity could be initiated by adding very small amounts of lysine (final concentration $3\cdot3 \times 10^{-5}$ M).

In the light of these experiments it seemed that cadaverine, the end-product of the reaction, might be the substance responsible for the de-adaptation. To test this, adapted cells were washed and incubated aerobically with and without cadaverine (10^{-4} M) under various conditions. From Fig. 7 it will be seen that: (*a*) in the control (glucose only) there was the usual slow fall in activity; (*b*) this was greatly accelerated by cadaverine; (*c*) cadaverine in the absence of glucose had no effect; (*d*) sodium azide (10^{-3} M) stabilized the decarboxylase activity. It was found that cadaverine was active in small amounts and an effect was shown by solutions as dilute as 10^{-6} M. To determine whether this de-adaptation was due to loss of apo-enzyme or of co-factor, cells were adapted in the usual way, washed, and then de-adapted by incubation with glucose and cadaverine. Lysine decarboxylase activity was measured both in intact cells and in cells pre-treated with toluene; activity in the latter was measured in the presence of pyridoxal phosphate. It was found (Table 2) that the apo-enzyme is stable during the de-adaptation, and the loss of activity is presumably due to loss of the co-factor. This finding exhausts the last of the explanations considered for the differences between aerobic and anaerobic induction.

Table 2. *Activity of intact cells compared with their apo-enzyme content during adaptation and de-adaptation*

Cells were adapted by incubation with lysine and glucose, washed and de-adapted by treatment with glucose and cadaverine (10^{-4} M) for 100 min.

	Intact cells	Apo-enzyme
Basal activity:	4	3
Adapted:	47	122
De-adapted:	30	118

Diamine oxidase

Since the de-adaptation of the intact cells seemed to depend on active metabolism (requiring glucose and prevented by azide), it was considered that the phenomenon might be due to the induced formation of another enzyme, possibly diamine oxidase, by the cadaverine, with a consequent sharing of the cell's pyridoxal phosphate by the two enzymes. An attempt was made to see whether diamine oxidase formation could be induced by cadaverine, and, secondly, to examine the effect of some other substrates of diamine oxidase on adapted cells.

Washed cells were incubated with glucose and cadaverine (0·005 M) at pH 5·5. Samples were taken at intervals during 2 hr. After washing, the cells were suspended in phosphate buffer at pH 7·0, and 2·5 ml. of the suspension added to Warburg flasks. Cadaverine dihydrochloride (0·5 ml. of 0·033 M solution) was used as test substrate and oxygen uptake measured at 30°. The occurrence of oxidation could not be demonstrated in any of the samples even with 35 mg. dry weight of bacteria in the manometers.

Following this, the effect of putrescine and histamine (both 0·0005 M) on adapted cells was compared with that of cadaverine at the same concentration. Putrescine had an action similar to that of cadaverine while histamine was inactive (Fig. 8).

Lysine analogues

An attempt was made to find an inducer which was not attacked by the enzyme, so that the complication introduced by the production of cadaverine during induction would be avoided. The following compounds were tested both for ability to induce enzyme synthesis and ability to inhibit induction by lysine: L-ϵ-*N*-acetyllysine, L-ϵ-*N*-benzoyllysine, α-amino-ϵ-hydroxycaproic acid and α-ϵ-diaminoheptanoic acid. The two last were synthetic products and the optical isomers had not been resolved. All these compounds were inactive.

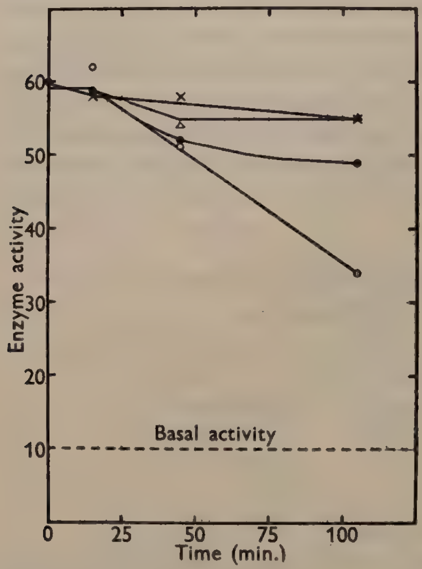


Fig. 7

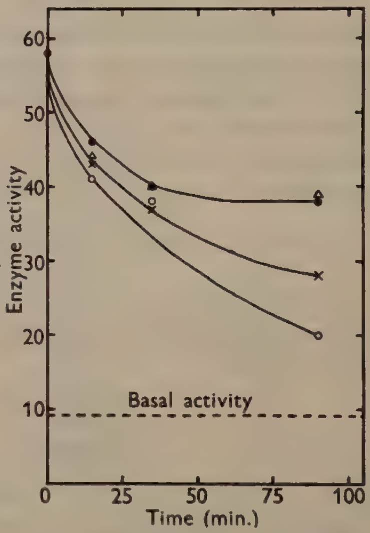


Fig. 8

Fig. 7. Effect of cadaverine on rate of de-adaptation. Adapted cells were washed and incubated as follows: ●—●, 1% glucose; ×—×, cadaverine (10^{-4} M); ○—○, glucose + cadaverine; △—△, glucose + cadaverine + NaN$_3$ (10^{-3} M). Activity measured on intact cells.

Fig. 8. Effect of cadaverine, putrescine and histamine on rate of de-adaptation. Adapted cells were washed and incubated as follows: ●—●, 1% glucose (control); ○—○, glucose + cadaverine (5×10^{-4} M); ×—×, glucose + putrescine (5×10^{-4} M); △—△, glucose + histamine (5×10^{-4} M). Activity measured on intact cells.

DISCUSSION

It will be of interest to consider, first, the induced synthesis of lysine decarboxylase from the point of view of its rate of formation. In general, the curve of rate of synthesis of an induced enzyme might have the following different forms depending on the experimental conditions (see Mandelstam, 1952):

(*a*) *Exponential*—when the action of the induced enzyme on its substrate provides energy or material for the formation of more enzyme, that is, when the induced enzyme is not formed under what Monod & Cohn (1952) have called conditions of 'gratuity'. Similarly, an exponential rate would be expected when the cells are growing in the presence of the inducer.

(*b*) *Linear*—when the enzyme is produced under conditions of 'gratuity' from a relatively large pool of material.

(*c*) *Falling*—when the pool of material is restricted.

The linear rate of appearance of lysine decarboxylase is therefore an indication—though not a proof—that the enzyme is being produced in conditions of 'gratuity' and, presumably, from a pool of material which is large relative to the amount of enzyme synthesized. With regard to the lag period found in the aerobic, but not in the anaerobic, experiments no definite explanation has been found for the phenomenon. The lag usually lasted for about 10 min., but was often shorter and occasionally absent altogether. It can, perhaps, be ascribed to the retarded diffusion of lysine into the cell under aerobic conditions.

Considering, now, the adaptive behaviour of the intact cells, rather than their apo-enzyme content, it seems that the activity found at any one time is the resultant of two opposite processes: (i) an adaptive process caused by lysine; (ii) a de-adaptive process caused by cadaverine. The latter only becomes manifest when the concentration of lysine in the medium is no longer sufficient to stabilize the decarboxylase system. It was found, in experiments not quoted here, that, by adding small amounts of lysine at suitable intervals, adaptation and de-adaptation could be made to occur repeatedly.

The de-adaptation is apparently due to loss of pyridoxal phosphate, since the apo-enzyme is quite stable. A possible explanation seemed to be the induced formation of an enzyme which oxidizes cadaverine, with consequent sharing of a limited amount of the co-factor by the two enzymes. However, the evidence for this has not been found, since oxidation of cadaverine could not be demonstrated. The effect of cadaverine on pyridoxal phosphate has not been further investigated, and it is still possible that another enzyme is being induced which appropriates some of the pyridoxal phosphate. Alternatively, the presence of cadaverine may lead to an actual destructive loss of pyridoxal phosphate unless lysine is present to stabilize the decarboxylase. A stabilizing effect of substrate on pyridoxal phosphate has been found with ornithine decarboxylase in a *Lactobacillus* sp. (Rodwell, 1953).

As stated in the introduction, the object of these experiments was to seek confirmatory evidence for either the 'mass action' or the 'catalyst' theories of enzyme induction. It may be useful to consider to what extent the facts are in keeping with each of these concepts. Restricting the discussion to the synthesis of the apo-enzyme, the facts consistent with the 'mass action' theory are as follows: (1) the pH curves for enzyme induction and enzyme activity correspond closely, having similar peak values and similar form; (2) the rate of anaerobic synthesis is directly proportional to the degree of saturation of the enzyme by substrate. Both facts can be regarded as support for the idea that the concentration of the enzyme-substrate complex is the determining factor in the synthesis of the enzyme.

Against the theory is the fact that when enzyme production takes place aerobically, the rate is maximal even when the enzyme is largely unsaturated. The possibility that lysine is concentrated by the bacteria, so that the enzyme is saturated even at low external concentrations of substrate, has been examined and found to be untenable. Furthermore, it follows from the 'mass action' theory that, on removal of the substrate, the induced apo-enzyme should disappear at least as rapidly as it was formed; in fact, the apo-enzyme is stable in the absence of lysine. In view of these facts it is apparent that the 'mass action' theory does not provide an adequate description of the system and could be retained only by making a number of *ad hoc* assumptions.

From the standpoint of the 'catalyst' theory, the results of the aerobic induction experiments are consistent and so is the stability of the apo-enzyme in the absence of inducer. The anomalous facts are, first, the close correlation between the rate of anaerobic synthesis and the saturation of the enzyme, and secondly, the equally close correlation between the pH curves for enzyme induction and enzyme activity. To make the 'catalyst' theory fit these facts it would be necessary to postulate *ad hoc* properties either for the enzyme-synthesizing mechanism or for the receptor. Thus, it could be assumed that induction is 'catalytic' but that, anaerobically, the enzyme-synthesizing mechanism depends on the decarboxylation of lysine for energy or material; that is, the enzyme is not being produced under conditions of 'gratuity'. This possibility has already been discussed and must be considered unlikely as it would entail an exponential rate of synthesis.

A further possibility is that lysine, as such, is required for the synthesis of new enzyme. If this were so, additional assumptions would have to be made to account for the pH curves, for the difference between aerobic and anaerobic induction by small amounts of lysine, and for the correlation between enzyme saturation and the anaerobic rate of synthesis. The assumptions that would have to be made are: (*a*) synthesis of enzyme is optimal at that pH at which the enzyme is most effective; (*b*) anaerobically lysine is the limiting factor in enzyme synthesis but aerobically it is needed only in traces if at all; (*c*) the enzyme-synthesizing mechanism, anaerobically, has exactly the same affinity for lysine as the enzyme itself. While Gale's work on the amino acid decarboxylases (1940) has shown that the first of these assumptions is justifiable, the second and third are much less probable and would, in any case, be difficult to verify experimentally.

Alternatively, the 'catalyst' theory could be retained by making the following assumptions about the receptor. (*a*) Aerobically the receptor has a relatively high affinity for lysine so that it is saturated at a concentration where the enzyme is almost completely unsaturated; anaerobically the affinity falls to a lower order altogether and the receptor now has a saturation curve that is virtually the same as that of the enzyme. It would have to be assumed that this similarity is fortuitous. (*b*) Both aerobically and anaerobically the affinity of the receptor for lysine is affected by pH in the same way as that of the enzyme and, again, the agreement would have to be considered fortuitous.

The position therefore seems to be that both the 'mass action' and the 'catalyst' theories are in accordance with some of the experimental facts, but that neither provides a complete explanation.

I wish to thank Miss R. Coyle for invaluable technical assistance and I am indebted to Roche Products Ltd. for a generous gift of pyridoxal phosphate.

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The Isolation and Description of two Marine Micro-organisms with Special Reference to their Pigment Production

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SUMMARY: Two coryneform organisms (A1032 and A1062) isolated from the skin mucus of Arctic cod, are described in detail. Strain A1032 may be a new species; strain A1062 is a strain of *Corynebacterium erythrogenes.*

Three carotenoid pigments, all with very similar absorption spectra, were found in both strains. Two of these pigments were identified as neoxanthin and sarcinaxanthin. The third pigment has not been described previously and has been named corynexanthin; it is characterized by an extremely high adsorptive power. The three pigments occur in different relative amounts in the two strains. Diphenylamine has no qualitative effect on the pigments produced, but does tend slightly to inhibit the amount produced.

Strain A1062 on certain media (notably blood agar base, Oxoid), produces large amounts of coproporphyrin III and smaller amounts of pentacarboxy-, tricarboxy-, and dicarboxy-porphyrins. Strain A1032 does not produce porphyrins.

Most bacteria isolated from the skin slime of various marine teleost fish are Gram-negative rods (Shewan, 1949). Organisms of the genus *Corynebacterium*, however, form an important part of the flora of shark which inhabit Australian waters (Wood, 1950), and they have also been identified in the bacterial flora of cod (*Gadus morrhuae*, L.) caught off Bear Island (Shewan & Hodgkiss, 1951). According to Jensen (1952) these corynebacteria should be placed in his section *Corynebacterium sensu lato.*

The first part of this paper contains a detailed description of two such strongly chromogenic coryneform organisms isolated during a survey of the normal bacterial flora of Arctic cod undertaken with a view to identifying organisms concerned in the spoilage of fish (Shewan & Hodgkiss, unpublished observations). The second section describes the pigments elaborated by these bacteria.

A characteristic of marine bacteria is that a large proportion of them are coloured (ZoBell, 1946); for example, ZoBell & Feltham (1934) examined several thousand colonies growing on agar inoculated with sea water or marine mud, and found that 69·4 % were chromogenic. Later ZoBell & Upham (1944) described fully twenty coloured species. Many species of marine bacteria which spoil fish are also chromogenic; the considerable literature on this subject has been summarized by ZoBell (1946). In spite of the availability of considerable amounts of material, no investigations into the nature of the pigmentation of marine bacteria have been reported. There is one report of carotenoids in a halophilic, but not marine, member of the Thiorhodaceae, *Chromatium okenii*, which is reported to contain, apart from bacteriochlorophyll, the carotenoid α-bacteriopurpurin (Lévy, Teissier & Wurmser, 1925). This pigment is one of two (α- and β-bacteriopurpurin) obtained by Molisch (1907) from *Bacterium rubescens*. α-Bacteriopurpurin also occurs in *Flavobacterium arborescens* (Sobin & Stahly, 1942), in *Bacterium halobium* (Lederer, 1938) and possibly in traces in *Rhodospirillum rubrum* (Goodwin & Osman, 1954); it is possibly demethylated rhodoviolascin (spirilloxanthin) (Karrer & Solmssen, 1935; Polgár, van Niel & Zechmeister, 1944). Although the experimental information is not complete, it is almost certain that all the purple sulphur and non-sulphur bacteria contain carotenoids along with bacteriochlorophyll (see Goodwin, 1952*a*, for full details).

Little is known about the general factors which control pigmentation in marine bacteria. ZoBell & Upham (1944) found that pigmentation was increased when the bacteria were grown in sea water enriched with Bacto-tryptone, neopeptone and beef extract at a low temperature (4°). ZoBell (1946) also quoted unpublished work by Upham which indicated that infusions of fish, octopus, mussel and other marine animals stimulated pigment production.

With regard to pigments in non-marine coryneform organisms, previous early reports indicated that β-carotene and unidentified xanthophylls were present in *Corynebacterium* spp., including *C. diphtheriae* (Skinner & Gunderson, 1932; Baumann, Steenbock, Ingraham & Fred, 1933; Chargaff, 1933). Recently, Starr & Saperstein (1953) showed that the plant pathogen *C. poinsettiae* produces spirilloxanthin and lycoxanthin when grown on thiamine-low medium, and lycoxanthin + cryptoxanthin, with only small amounts of spirilloxanthin, on a thiamine-rich medium.

THE ORGANISMS

The two organisms to be described (A1032 and A1062) were primary isolations from the skin slime of *Gadus morrhuae* on horse-heart infusion agar cultures incubated at 20°.

*Organism A*1032

Morphology. On horse-heart infusion agar the cells are slender, straight rods measuring *c.* $0{\cdot}4\mu. \times 2{\cdot}0$–$3{\cdot}0\mu.$ and are usually very uniform in size. They are non-motile, Gram-positive, non-acid-fast and do not form spores. Angular and palisade arrangements of the cells occur.

Colony appearances. After 5 days of incubation on horse-heart infusion agar at 20° colonies are circular, 1·5 mm. in diameter, with an entire margin and a convex to pulvinate smooth surface. They are semi-translucent, lemon-yellow in colour.

Growth in fluid media. At first a uniform turbidity is produced, growth then becomes flocculent and a yellow sediment is formed. Pellicle formation does not occur.

Temperature range. The optimal temperature for growth is 20–25°. No growth occurs at 37° or at 0°. Growth at 5° is moderate.

Biochemical characteristics. Acid is produced rapidly from glucose and maltose, and slowly from sucrose and mannitol. Lactose and dulcitol are not fermented and starch is not hydrolysed. Gelatin and Loeffler's serum are not liquefied. Nitrate and trimethylamine oxide are not reduced. Ammonia is not produced from peptone or from urea. Indole, methyl-red and Voges-Proskauer tests are negative. Litmus milk shows no change after incubation for 6 weeks. Sodium alginate and cellulose are not attacked.

Classification. It is considered that this organism must be classified as a *Corynebacterium* sp. and in accordance with the recommendations of Jensen would be placed in his section '*Corynebacterium sensu lato*'. It does not resemble any of the species listed in *Bergey's Manual* (1948), and on further investigation may prove to be a new species.

Organism A 1062

Morphology. On horse-heart infusion agar the cells are rods measuring *c.* 0·6–0·8 μ. × 4·0–5·0 μ. In fluid media the cells are often longer (up to 8·0 μ.). They may be straight or slightly curved, sometimes having a very angular appearance; a characteristic feature is the uneven thickness of the cell. False branching and 'budding' occurs. The organisms are non-motile, Gram-positive, non-acid-fast, non-spore forming.

Colony appearances. After 5 days of incubation at 20° on horse-heart infusion agar colonies are circular, 2·0–3·0 mm. in diameter, with an entire margin and a smooth moist convex to pulvinate surface. They are opaque and lemon-yellow in colour. Older cultures show rough variants and have a dried matt surface. A characteristic feature is the production of a red fluorescent pigment which diffuses into the medium when the organism is grown on certain media (see next section).

Growth in fluid media. At first a uniform turbidity and a thick yellow pellicle is produced. Usually the pellicle disintegrates (upon being disturbed or under its own weight) and growth becomes flocculent with a yellow flocculent sediment.

Temperature range. The optimal temperature for growth is 20–25°; growth does not occur at 37°. At 0° there is an appreciable growth after 7–10 days of incubation.

Biochemical activities. Glucose, lactose, sucrose, maltose, dulcitol and mannitol are not fermented and starch is not hydrolysed. Gelatin stabs show slow infundibuliform liquefaction, which later becomes stratiform. Loeffler's serum is not liquefied. Ammonia is produced from urea and from peptone. Nitrates are reduced to nitrites. Indole, methyl-red and Voges-Proskauer tests are negative and trimethylamine oxide is not reduced. Litmus milk becomes strongly alkaline and a clot forms. Sodium alginate and cellulose are not attacked.

Classification. This organism is considered to be a marine variant of *Corynebacterium erythrogenes* Kisskalt & Berend, 1918 (*Bacterium erythrogenes*

Lehmann & Neumann). It differs from the original description only in the size of the cells, and in the cultural conditions under which the red pigment is produced.

THE PIGMENTS

METHODS

Cultures. The two coryneform organisms A1032 and A1062 were cultivated mainly on blood agar base at room temperature (about 15°), but a number of other standard media were also used. The *Sarcina lutea* culture used to produce reference carotenoids was a strain (21) kindly provided by Dr H. K. King. It was grown on nutrient broth at 37°.

Extraction, separation and characterization of the pigments

Carotenoid pigments. The bacterial cells were scraped off the agar and shaken with methanol on a warm water-bath (50–60°) for 2–3 min.; this extracted almost all the pigment into the methanol, which was decanted after allowing the residue to settle. The residue was similarly extracted once with methanol, and to the combined extracts was added an equal volume of diethyl ether, followed by water until two layers were formed. The upper ethereal layer, containing all the pigment, was freed from methanol by washing 3 times with small volumes of water. The ether was removed *in vacuo* at room temperature and the residue dried by adding a few drops of absolute ethanol and then blowing dry with a stream of N_2 on a warm water-bath. The dry residue was dissolved in a very small volume of diethyl ether which was then diluted with light petroleum (the fraction boiling between 40 and 60° was used throughout this investigation) to make an appropriate volume (5 ml.) for chromatography.

It is important to note that the procedure usually used for the preliminary extraction of carotenoids from micro-organisms (Garton, Goodwin & Lijinsky, 1951; Goodwin, 1952*b*) was not applicable in the case of these coryneform bacteria. The usual method involves grinding the cells to a fine dry powder with acid-washed silver sand and anhydrous Na_2SO_4 and then extracting the pigments with warm diethyl ether or acetone. With the coryneform bacteria, no solvent investigated would extract the pigments from the dry powder; even methanol containing 5% (v/v) glacial acetic acid was ineffective. The reason for this is, as will be seen later, partly due to the very great adsorptive power of the constituent carotenoids which remained firmly attached to the solid Na_2SO_4; the failure to extract the pigments with methanol + acetic acid is, however, somewhat inexplicable. It is obviously important that the failure to extract pigments from a coloured Na_2SO_4-dried bacterial powder with the usual fat solvents should not be construed as indicating the absence of carotenoids until other extraction procedures have been examined.

Carotenoids from Sarcina lutea. The cells of *S. lutea* grown on nutrient broth were harvested by centrifugation, and the carotenoids extracted from the cell mass by the method just described for the coryneform organisms.

*Porphyrins from organism A*1062. The porphyrins which were excreted into the solid medium were extracted, after the removal of the bacteria, by warming the medium on a water-bath until just molten and then shaking it with diethyl ether containing 5 % (v/v) glacial acetic acid.

Separation and identification of carotenoids. The carotenoids were chromatographed using three types of adsorption columns: (*a*) Alumina (Grade 'O'; P. Spence and Co., Widnes) deactivated with methanol according to the method of Goodwin & Srisukh (1949). (*b*) $CaCO_3$ of analytical grade previously dried at 140° for 2 hr. and kept in a desiccator until used. It is important to use analytical $CaCO_3$ because the laboratory grade contains $Ca(OH)_2$, which is much more adsorptive than $CaCO_3$ (Lederer, 1952). (*c*) Powdered cellulose (Whatman, Standard Grade). The solvents used for elution of the pigments were light petroleum, diethyl ether and ethanol.

The absorption spectra measurements were carried out on either a Beckman photoelectric spectrophotometer (Model DU) or the corresponding Unicam instrument (SP 500).

Saponification of the lipid extracts was carried out according to the method of Goodwin & Morton (1946). The phase tests on the pigments were carried out by shaking a light petroleum solution of the pigment with methanol containing 10 % (v/v) of water.

Identification of the porphyrins. The detailed examination of the porphyrins was kindly carried out by Prof. C. Rimington, F.R.S., after our preliminary examination of absorption spectra, fluorescence and behaviour on paper chromatography had demonstrated their presence.

Quantitative determination of the pigments. The amount of carotenoids present was determined assuming $E^{1\%}_{1\text{cm.}}$ ($\lambda_{\text{max.}}$) to be 2500. The porphyrins were determined using the value $E^{1\%}_{1\text{cm.}}$ (401 mμ.) 7400, reported by Todd (1949) for coproporphyrin III.

RESULTS

Pigments of coryneform organism A 1032

The carotenoid mixture, which the partition test showed to consist of xanthophylls containing at least two free hydroxyl groups, was first chromatographed on weakened alumina. The major part of the pigment was so tightly adsorbed that it was impossible to remove it from the adsorbent with any solvent. A small fraction (A) was eluted with ethanol. There was no sign of any colourless fluorescing polyenes such as phytofluene. On chromatography on either $CaCO_3$ or powdered cellulose, three fractions were obtained as described in Table 1. The most interesting fact about these three fractions is that they differ only in adsorptive power, their absorption spectra being almost identical.

The possibility existed that the pigment of zone C, being the most strongly absorbed, was the parent polyhydroxy compound and that the pigments of zones A and B were derivatives in which different numbers of hydroxyl groups had been esterified, for it is well known that xanthophylls can exist in nature in the esterified state (Goodwin, 1952*a*). This possibility was ruled out by saponifying a pigment extract and rechromatographing the resulting mixture. The separation obtained was qualitatively and quantitatively the same as before saponification, thus indicating that the zones A and B contained distinct pigments and not esters of pigment C.

Table 1. *The separation of the carotenoids of coryneform organism A* 1032 *on* $CaCO_3$ *and on powdered cellulose*

Zone	Colour	$CaCO_3$ (solvent system required to remove from column)	Powdered cellulose (solvent system required to remove from column)	Absorption spectral maxima in ether (mμ.)	Identification
A	Greenish yellow	Light petroleum	Light petroleum	414, 437, 467	Neoxanthin
B	Greenish yellow	Light petroleum +15–20 % (v/v) ether	Light petroleum +5 % (v/v) ether	414, 437, 467	Sarcinaxanthin
C	Greenish yellow	Ether+5 % ethanol	Ether	415, 438, 467	New pigment (corynexanthin)

Zone A (Table 1), which was the same as zone A obtained from alumina, had an absorption spectrum (both in position and shape) and adsorptive power almost identical with those of neoxanthin and was indistinguishable from a specimen of this pigment obtained from a number of algae (Goodwin & Jamikorn, unpublished observations). Neoxanthin was first obtained in small amounts from leaves by Strain (1938).

The pigment of zone B was provisionally identified as sarcinaxanthin. Consequently, the pigments of a strain of *Sarcina lutea* were examined. Chromatography on $CaCO_3$ yielded essentially one pigment which was eluted with light petroleum containing 15–20 % (v/v) ether. Very small amounts of another pigment with the same absorption spectrum as the main fraction were removed with ether containing 5 % ethanol; it was probably the same as pigment C in the coryneform organisms. No carotenes were observed. The main fraction which is undoubtedly sarcinaxanthin (Takeda & Ohta, 1941; Sobin & Stahly, 1942) had the same absorption spectrum as pigment B, and on carrying out a mixed chromatogram with pigment B and sarcinaxanthin on $CaCO_3$ no separation could be obtained. These observations, combined with the fact that no other carotenoids are known with the same absorption spectrum and the same adsorptive power (see Goodwin, 1952*a*), make it certain that pigment B is sarcinaxanthin.

Pigment C is entirely new, for no carotenoid is known with such very high adsorptive power; astaxanthin and fucoxanthin, which are bordering on the same degree of adsorptive power, have entirely different spectral and general properties (Karrer & Jucker, 1950; Goodwin, 1952*a*). It is proposed to name this new carotenoid corynexanthin. The absorption spectrum of corynexanthin in various solvents is given in Fig. 1, and the exact absorption maxima of neoxanthin, sarcinaxanthin and corynexanthin in various solvents in Table 2. It should be emphasized that, as the shapes of the spectra of these three pigments are almost identical, it is impossible to distinguish them solely by spectroscopic methods. The same three pigments were produced by coryneform organism A 1032 on all the media examined.

Pigments of coryneform organism A 1062

The presence of the same three carotenoid pigments obtained from organism A 1032 were demonstrated in organism A 1062 by using the same techniques. The relative amounts of the three pigments in the two organisms were determined; Table 3 shows the result of a typical experiment. It will be seen that whilst neoxanthin existed to the same extent in both strains there was a considerable difference in regard to sarcinaxanthin and corynexanthin. Sarcina-

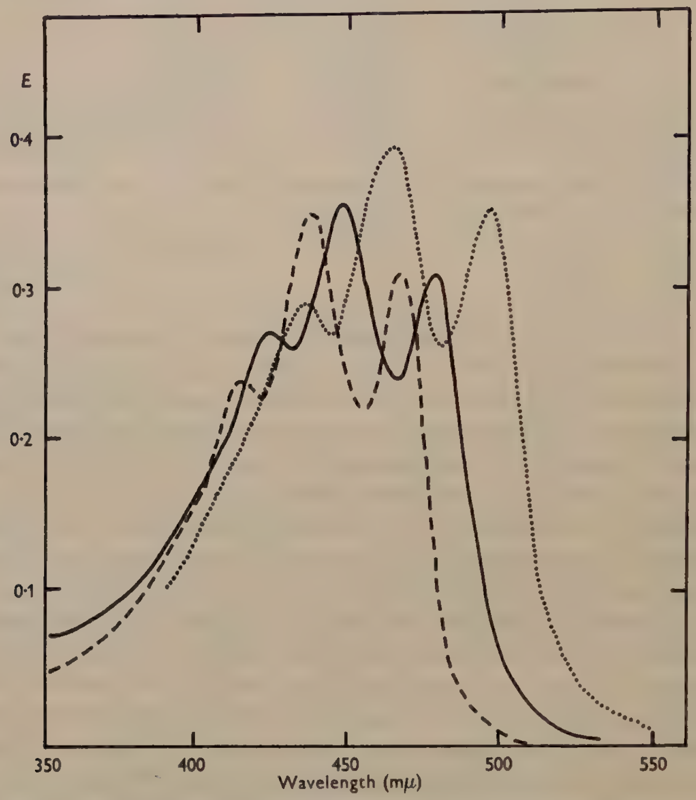

Fig. 1. The absorption spectrum of corynexanthin in various solvents. ----, diethylether; ——, chloroform; ·····, carbon disulphide.

Table 2. *The absorption spectral maxima (mμ.) of the carotenoid pigments in the coryneform organisms A* 1032 *and A* 1062 *in various solvents*

	Solvent			
	Diethyl ether	Ethanol	Chloroform	Carbon disulphide
	Spectral maxima (mμ.)			
Neoxanthin	414, 437, 467	415, 437, 467	422, 447, 478	433, 458, 489
Sarcinaxanthin	414, 437, 467	417, 439, 469	422, 448, 476	436, 467, 497
Corynexanthin	415, 438, 467	416, 440, 469	423, 447, 478	435, 466, 497

xanthin was the major component in organism A 1032 and corynexanthin in organism A 1062 (Table 3). The same relative amounts of carotenoids were always produced on the different media tested.

Table 3. *The relative amounts of the carotenoid pigments in the coryneform organisms A 1032 and A 1062*

Pigment	Coryneform organism A 1032	Coryneform organism A 1062
	% of total pigments present	
Neoxanthin	21·3	26·3
Sarcinaxanthin	73·3	23·5
Corynexanthin	5·4	50·2

The porphyrins produced by organism A 1062

It was found that organism A 1062 when grown on Lab-Lemco agar (Oxoid) or blood agar base (Oxoid) formed a brownish red pigment in the medium. This pigment could be extracted from the medium, after growth, by diethyl ether containing 5 % (v/v) glacial acetic acid. It fluoresced bright pinkish red in ultraviolet light and exhibited an absorption spectrum with the position and shape of bands corresponding to coproporphyrin III ($\lambda_{\text{max.}}$, 624, 595, 571, 529, 497, 389, 271 mμ. in acid ethanol; compare 623, 596, 531 and 497 mμ. recorded by Lemberg & Legge, 1949). Paper chromatography according to the method of Nicholas & Comfort (1949) indicated one major pigment with approximately the same R_F value as coproporphyrin III; a small amount of brown material was also present. Further examination of this pigment was carried out by Prof. Rimington and his colleagues who reported: 'Chromatography by Eriksen's method in 2:6-lutidine showed that it was mainly coproporphyrin III, but it also contained some pentacarboxylic porphyrin and some tricarboxylic porphyrin, both giving a fairly strong spot. Examined by the Chu method, the pigment appeared to move as coproporphyrin III. It would appear therefore that the pigment produced is, in fact, mainly coproporphyrin III.'

Examination of porphyrin formation on various laboratory media by organism A 1062 showed that it occurred only with growth on blood agar base (Oxoid) and Lab-Lemco (Oxoid). No formation of porphyrin was observed on the following media: agar (British Drug Houses Ltd., or Oxoid) + peptone (Evans or Oxoid); agar + peptone + yeast extract; nutrient broth (Oxoid); nutrient broth + sterile blood (Evans); nutrient broth + yeast extract, although growth was good on all these media except on agar + peptone + yeast extract. Although no porphyrin was formed in the medium containing blood, the haem appeared to be metabolized because the medium lost its red colour soon after inoculation with the bacteria. When grown on blood agar basal medium made in our laboratory from the commercially available constituents, Lab-Lemco, peptone and agar (all Oxoid), no porphyrin was produced, although the bacterium grew normally. Through the kindness of Messrs Oxo Ltd. we were able to examine the crude meat extract, used in the manufacture of blood agar base, which is purified before being marketed as Lab-Lemco. Using this with Oxoid peptone and agar, a blood agar base was produced on which organism A1062 did produce porphyrin, although in smaller amounts than when grown on the commercial blood agar base. The replacement of Oxoid peptone by the peptone syrup used in the production of commercial blood agar base had no stimulatory effect on porphyrin production. Porphyrin was also synthesized in the medium used to test for ability of the organism to reduce trimethylamine oxide (Wood & Baird, 1943). No porphyrin was formed following the removal of trimethylamine oxide from the medium or its replacement by betaine, dimethylamine or trimethylamine.

Organism A1032 produced no porphyrin on any medium examined.

Quantitative experiments on pigment production

The pattern of pigment synthesis by organism A1062 was investigated and the results are shown in Table 4. The production of carotenoids and of porphyrin was maximal between the fourth and sixth days of incubation. The porphyrin disappeared quite quickly from older cultures, the carotenoids much less quickly. Some of both kinds of pigment, however, remained even in very old cultures which were still slightly yellow, and in the medium which fluoresced slightly pink.

Table 4. *Carotenoid and porphyrin formation by organism A* 1062 *at various stages of growth*

Amounts produced on 10 ml. blood agar medium. Incubation temperature 15°. Values are mean of three tubes.

	Media			
	Blood agar base	Blood agar base + diphenylamine (1/35,000)	Blood agar base	Blood agar base + diphenylamine (1/35,000)
Age of culture (days)	Total carotenoids (µg.)		Porphyrins (µg.)	
2	0·9	—	5·0	—
4	2·4	0·4	15·7	3·1
6	2·2	—	15·9	—
8	2·1	1·6	8·5	10·2
10	1·9	—	5·5	—
12	1·8	1·4	4·9	10·1

The action of diphenylamine

Diphenylamine is a specific inhibitor of carotenogenesis in *Mycobacterium phlei* (Turian, 1950) and *Phycomyces blakesleeanus* (Goodwin, 1952*b*). In each case, although the synthesis of coloured polyenes (carotenoids) is inhibited, there is a concomitant increase in the synthesis of related colourless polyenes such as phytoene, phytofluene, etc. The effect of diphenylamine on carotenoid production by organism A1062 is shown in Table 4, which indicates that the amount produced/tube was decreased and that this effect was also apparent on the synthesis of porphyrin. From the appearance of 8-day and older cultures, when growth covered the agar slopes, this effect did not appear to be due to an inhibition of growth. Growth rate was, however, considerably decreased in the presence of diphenylamine; there was not sufficient growth to carry out an analysis after 2 days incubation and only just sufficient at 4 days. More important from the point of view of carotenoid synthesis is the fact that chromatographic analysis of the carotenoids extracted from diphenylamine-containing cultures demonstrated the presence of the three normal pigments only; there was no trace of the more saturated polyenes which one might have expected to have been produced under the influence of diphenylamine. Diphenylamine-containing cultures of organism A1032 behaved in the same way as the corresponding cultures of strain A1062.

DISCUSSION

The carotenoids

The fact that the three xanthophylls present in organisms A1032 and A1062 have almost identical absorption spectra but different absorptive powers, indicate that they must contain the same chromophoric grouping but different numbers of polar groupings (hydroxyl, keto, epoxy, or carboxyl). It is possible from the information available to draw some conclusions about the structure of these pigments, especially in the sense of eliminating unlikely structures. Assuming the pigments are C_{40} carotenoids (as are almost all naturally occurring carotenoids) the following can be ruled out: (*a*) keto-groups, because the characteristic property of a ketonic carotenoid, e.g. astaxanthin or echinenone, is its single banded spectrum (Goodwin & Srisukh, 1949; Goodwin & Taha, 1950); (*b*) carboxyl-groups, because the pigments show no acidic properties; (*c*) the 5, 8-epoxy-derivatives of α and β carotenes because their spectral maxima are at lower wavelengths than are those of the present pigments (see Goodwin, 1952*a*; Karrer & Jucker, 1950).

As far as present knowledge goes, only two possibilities remain: (i) the pigments are polyhydroxy—partly hydrogenated derivatives of α-, β- or γ-carotenes or lycopene; or (ii) hydroxy derivatives of 5:8-epoxy-γ-carotene.

With regard to possibility (i), the pigments must contain three or more hydroxyl groups because they are very much more strongly absorbed than the dihydroxy pigments lutein and zeaxanthin. They must be more saturated than the carotenes mentioned because their absorption spectral maxima are at lower wavelengths. The strong definition of the absorption spectra of the pigments suggests that they are derivatives of lycopene. If this be so, then the difference of 33–35 mμ. between the positions of the absorption maxima of lycopene and those of the present pigments indicates that they would be tetrahydrolycopene derivatives. This would make them derivatives of the known pigment neurosporene (Haxo, 1949). The absorption maxima of neurosporene agree almost exactly with those of the present pigments, but the shape of its curve is somewhat different. Although the definition of the curves of

α- and β-carotene is much less than that of the present pigments, they cannot be ruled out as parents of these pigments because it is not known what the effect of partial saturation is on the shape of the α- and β-carotene curves.

With regard to possibility (ii): 5:8-epoxy-γ-carotene has not yet been described as a natural product, but from the known properties of 5:8-epoxides (Karrer & Jucker, 1950), the absorption spectrum of such a compound would be very similar to that of the coryneform pigments although the shape might be different. A further point against a 5:8-epoxy structure is that these compounds give a characteristic blue coloration with ethereal HCl; the coryneform pigments give no such colour.

The failure of diphenylamine to have any effect on the types of polyene synthesized by these coryneform bacteria is further demonstration that the mechanism of carotenoid synthesis in different organisms must vary considerably. The action of diphenylamine on *Euglena gracilis* v. *bacillaris* (Goodwin & Jamikorn, unpublished observations) and *Rhodopseudomonas spheroides* (Goodwin, Land & Osman, unpublished observations), is similar to that on the coryneform pigments, but in *Mycobacterium phlei* (Turian, 1950; Turian & Haxo, 1952; Goodwin & Jamikorn, unpublished observations), *Phycomyces blakesleeanus* (Goodwin, 1952*b*; Goodwin, Jamikorn & Willmer, 1952) and *Rhodospirillum rubrum* (Goodwin & Osman, 1954), the synthesis of the carotenoids is inhibited whilst that of the colourless polyenes is stimulated.

Porphyrin synthesis by organism A 1062

It is important to emphasize the very limited conditions under which porphyrin formation occurred with this organism. It occurred maximally only on commercially prepared Oxoid Lab-Lemco agar or blood agar base. These media, when prepared in the laboratory from commercially available components permitted porphyrin formation only when the crude meat extract, normally incorporated into the commercial complete media, was used instead of the purified Lab-Lemco. When this purified Lab-Lemco was used porphyrin formation did not occur. It must be concluded that not only does the purification of the meat extract remove a factor necessary for porphyrin formation but that the dehydration of the combined crude meat extract peptone syrup agar mixture to produce the commercial blood agar base, liberates or produces more of this unknown factor, for porphyrin formation was greatest on the commercially produced preparations. Whether this factor is related to trimethylamine oxide, which also stimulates some porphyrin formation, remains for further investigations to decide.

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The Effect of Ribonuclease on Phage-Host Interaction

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SUMMARY: Pancreatic ribonuclease inhibits the multiplication of a Rhizobium bacteriophage in liquid bacterial cultures by preventing permanent combination between phage and host. The addition of ribonuclease after phage has already combined with the bacteria does not prevent phage from multiplying, but it does decrease the rate of multiplication; the enzyme also interferes with multiplication of the bacteria. Experiments with ribonuclease in conjunction with chymotrypsin suggest that phage and bacteria unite in the presence of ribonuclease but that the union is only transitory and ends by the release of still active phage. In the presence of chymotrypsin, however, the phage becomes inactive during this transient union.

The multiplication of a Rhizobium bacteriophage in liquid culture of its host bacterium is inhibited by both ribonuclease and chymotrypsin, although neither enzyme affects plaque formation on agar media (Kleczkowski & Kleczkowski, 1952, 1954). Nor do they affect the bacteriophage when mixed with it in the absence of its host. But the fact that ribonuclease inhibits the multiplication of the host cells, whereas chymotrypsin does not suggested that they might inhibit phage multiplication by different mechanisms. Chymotrypsin does not affect the combination between phage and bacteria, but affects a transient stage in the infection process occurring after combination, for if chymotrypsin is added 1 min. after the combination, infection and phage multiplication proceed normally (Kleczkowski & Kleczkowski, 1954). The work described in the present paper was done to find the stage or stages of the infection process modified by ribonuclease alone or in conjunction with chymotrypsin.

METHODS

Methods and materials were similar to those used previously (Kleczkowski & Kleczkowski, 1952, 1954). Ribonuclease, crystallized by the method of Kunitz (1940), was dissolved in 0·067 M-phosphate buffer (pH 7·2) and heated for 5 min. in a boiling water-bath. Any coagulum that appeared was removed by centrifugation. The results with a series of different unheated preparations were inconsistent, probably because the preparations contained different amounts of materials affecting the inhibition by ribonuclease of phage/host interaction. One such material is a proteolytic enzyme, and there may be others; heating under the above conditions destroys proteolytic activity without greatly impairing the ribonuclease activity (Kleczkowski, 1948).

RESULTS

Effect of ribonuclease on the interaction between phage and live bacteria

Table 1 shows that the presence of 0·1 % ribonuclease in a phage + bacteria mixture prevented phage multiplication during the first 3 hr. of incubation and greatly slowed it during 24 hr. (test no. 1). Like chymotrypsin, it had no apparent effect on the phage alone (test no. 3).

To test whether the ability of bacteria to interact with phage is altered by ribonuclease, bacterial cultures were incubated with 0·1 % ribonuclease for 3 hr. at 25°. The bacteria were then centrifuged down, suspended in fresh medium and phage added (test no. 5). The incubated bacteria did not support normal phage multiplication, although multiplication was less inhibited than when the medium contained ribonuclease (test no. 1). This result contrasts with that obtained when bacteria were incubated with chymotrypsin (Kleczkowski & Kleczkowski, 1954), for after removing chymotrypsin the bacteria interacted normally with the bacteriophage.

Table 2 shows that the presence of 0·1 % ribonuclease almost entirely prevented phage from combining with the bacteria, in conditions when without it about 90 % of the phage combined. Also, when phage was added to bacteria which had previously been incubated for 3 hr. with 0·1 % ribonuclease and then sedimented by centrifugation and re-suspended in fresh medium, only about 50 % of the phage combined with the bacteria so treated. This result also contrasts with that obtained with chymotrypsin which does not affect the combination between phage and its host bacterium (Kleczkowski & Kleczkowski, 1954).

Table 3 shows that incubation of the bacteria alone for 1 hr. in presence of 0·1 % ribonuclease made about 70 % of them unable to form colonies (compare tests nos. 1 and 2, Table 3). By contrast, as ribonuclease protects the bacteria from lysis by phage, 4 times as many bacteria formed colonies after incubation with phage + ribonuclease as after incubation with phage alone (tests nos. 3 and 4). Ribonuclease can therefore alter the bacteria enough to make them unable to multiply, and effects less drastic than this might suffice to make normal interaction with bacteriophage impossible and so protect bacteria from phage. These results with ribonuclease are again in contrast with those previously obtained with chymotrypsin which protects bacteria from lysis by phage without detectably affecting bacterial multiplication (Kleczkowski & Kleczkowski, 1954).

Ribonuclease might prevent combination between phage and bacteria because it affects a ribonucleic acid situated on the bacterial surface, possibly the ribonucleic acid which Harris (1953) found to be removed by ribonuclease from heat-killed bacteria. However, the effect of ribonuclease may not be enzymic but may be caused by electrostatic combination of the enzyme with a component of the bacterial surface.

To test whether ribonuclease affects the interaction between phage and bacteria after they have combined, the enzyme was added to a phage + bacteria mixture after it had been incubated at 25° for half an hour, by which time about 80 % of phage had combined with bacteria. After 3 hr. more incubation the concentration of phage (assayed by plaque count) increased to about 60 % of the concentration expected if all the combined phage had multiplied normally. Obviously ribonuclease did not prevent the phage already combined with bacteria from multiplying. There are three possible reasons for the slight

Table 1. *Effect of incubation of bacteria with ribonuclease on their ability to support phage multiplication*

Phage (0·5 ml. of phage stock culture diluted 10^{-5}) was added to each mixture of fluids. The mixtures were then incubated at 25° and samples were plated at stated intervals.

	Composition of mixtures (in ml.)					Phage concentration (plaques/ml.)		
Test no.	24 hr. bact. culture	Medium	1 % ribonuclease solution	Buffer	Further treatment of the mixtures before adding the phage	Initial	After 3 hr.	After 24 hr.
1	4	—	0·5	—	None	810	790	35×10^6
2	4	—	—	0·5		860	80,000	113×10^8
3	—	4	0·5	—		830	—	810
4	—	4	—	0·5		850	—	820
5	4	—	0·5	—	After 3 hr. at 25° bacteria centrifuged down* and suspended in 4 ml. medium + 0·5 ml. buffer	830	3,500	40×10^8
6	4	—	—	0·5		840	85,000	97×10^8

* At 10,000 r.p.m. for 20 min.

Table 2. *Effect of ribonuclease on combination between bacteria and phage*

0·4 ml. of a phage stock culture diluted 10^{-5} was added to each of the four fluid mixtures shown in the second column; these were then incubated for a further period of 1 hr. at 25° and then centrifuged.* The sediments were suspended in 4 ml. of the medium and phage assayed in the supernatant fluids and in the suspended sediments.

Composition of mixtures 6·4 ml. of a 24 hr. bacterial culture mixed with:	Further treatment of the mixtures after 3 hr. incubation at 25° before adding phage	Phage concentrations (plaques/ml.)	
0·8 ml. 1 % ribonuclease	3·6 ml. centrifuged* and sedimented bacteria suspended in 3·2 ml. medium + 0·4 ml. buffer	Supernatant	330
		Sediment	310
	3·6 ml. untreated	Supernatant	610
		Sediment	60
0·8 ml. buffer	3·6 ml. centrifuged* and sedimented bacteria suspended in 3·2 ml. medium + 0·4 ml. buffer	Supernatant	70
		Sediment	680
	3·6 ml. untreated	Supernatant	65
		Sediment	680

* For 20 min. at 10,000 r.p.m.

decrease in the yield obtained after 3 hr. incubation: the first is a decrease in the numbers of phage particles produced by individual cells; the second, an extension of the latent period and/or of the period of release of phage from bacteria, which normally take together about 3 hr. (Kleczkowski & Kleczkowski, 1954); the third, prevention of phage multiplication in some cells. The last possibility seems the least likely because the whole culture ultimately lysed.

Table 3. *Effect of ribonuclease on the ability of bacterial cells to multiply and form colonies*

The mixtures were incubated for 1 hr. at 25°, then diluted 10^{-5}, 1 ml. samples of the diluted fluids mixed with 9 ml. of 0·7 % agar medium (which was previously melted and cooled to 42°) and poured into Petri dishes. Bacterial colonies were counted after 5 days at 25°.

	Composition of mixtures (in ml.) 2 ml. of a 24 hr. bacterial culture +				
Test no.	1 % ribonuclease solution	Buffer	Phage stock culture (undil.)	Culture medium	Number of colonies/plate (mean of 3 plates)
1	0·5	—	—	2	30
2	—	0·5	—	2	100
3	0·5	—	2	—	20
4	—	0·5	2	—	5

Joint effect of ribonuclease and chymotrypsin on the interaction between phage and live bacteria

The joint action of ribonuclease and chymotrypsin on the interaction between phage and host was examined for two reasons: first, to see whether ribonuclease, by preventing phage and host from combining, protects the phage from being inactivated by chymotrypsin, which seems able to inactivate only after phage has combined with its host; secondly, observations on such mixtures provide a method of seeing whether ribonuclease separates phage from bacteria with which it has already combined. There seemed no method of doing this directly, because of the short period between combination and irreversible infection, whereas what is known of the action of chymotrypsin suggested it might prolong this period sufficiently to test whether ribonuclease can separate the two and permit recovery of free phage.

Table 4 shows that the addition of ribonuclease before or immediately after adding chymotrypsin to bacteria which were then mixed with phage, only slightly decreased the rate at which chymotrypsin inactivated the phage (cf. test nos. 2, 4 and 5, Table 4). Table 4 also shows that adding ribonuclease 3 hr. after mixing chymotrypsin, phage and bacteria did not affect the concentration of residual phage (cf. test nos. 2 and 6, Table 4). Thus, ribonuclease neither prevented phage and bacteria from combining nor separated any active phage that had already combined.

At first sight the results of the experiments using the two enzymes together seem to conflict with those where each was used separately. There is good evidence that chymotrypsin acts by inactivating phage which is combined

Table 4. *Joint effect of ribonuclease and chymotrypsin on phage/host interaction*

	Composition of mixtures (in ml.)					Order of adding constituents of mixtures to the bacterial culture which was always taken first. The constituents of the mixtures were added immediately one after another unless stated otherwise. All incubations were at 25°	Concentration of active phage (plaques/ml.)			
Test no.	48 hr. culture of bacteria	0·2 % chymotrypsin solution	1 % ribonuclease solution	Buffer	Phage stock culture diluted 10^{-5}		Initial	After incubation of 1 hr.	3 hr.	6 hr.
1	3	—	—	0·6	0·4	Buffer, phage	680	680	65×10^3	55×10^5
2	3	0·2	—	0·4	0·4	CTR, buffer, phage		170	30	10
3	3	—	0·4	0·2	0·4	RNase, buffer, phage		650	690	—
4	3	0·2	0·4	—	0·4	CTR, RNase, phage		—	80	—
5						RNase, 3 hr. incub., CTR, phage		290	—	—
6						CTR, phage, 3 hr. incub., RNase		—	30	12

CTR = chymotrypsin; RNase = ribonuclease.

with bacteria but has no effect on free phage (Kleczkowski & Kleczkowski, 1954). When ribonuclease was used alone (Table 2) there was also evidence that normal combination between bacteria and phage did not occur. Yet when chymotrypsin and ribonuclease are present together chymotrypsin still had its usual inactivating action. The most reasonable interpretation of these facts seems to be that in the presence of ribonuclease phage and bacteria do combine, and that during this phase the phage can be inactivated by chymotrypsin; unless chymotrypsin is present the combination is only transitory, and phage and bacteria separate before infection occurs. Alternative explanations for the results with the two enzymes together would be that chymotrypsin affects: (i) ribonuclease so that it does not have its usual action in preventing phage and bacteria from combining; or (ii) the bacteria so that ribonuclease no longer acts on them in a way that can prevent combination with phage. However, neither of these explanations seems very probable. Although chymotrypsin can hydrolyse ribonuclease (Kleczkowski, 1948), it does so very slowly, and in the conditions of these experiments the amount hydrolysed would be insignificant. Also, chymotrypsin does not remove from the bacteria the substance on which ribonuclease acts, because this is needed for infection between phage and bacteria and infection occurs normally after bacteria have been incubated with chymotrypsin.

The conclusion is that, in the presence of ribonuclease, phage and bacteria combine, but that the combination is broken up before infection occurs or the phage has been permanently altered. A bacterial ribonucleic acid which is not concerned with the initial combination between phage and bacterium, might be essential to some second step that makes combination irreversible. The hydrolysis of this ribonucleic acid by ribonuclease would prevent newly combined phage from being 'fixed' to the bacterial surface so that the combination could be transitory and apparently unaltered phage set free again. Chymotrypsin inactivates the phage during the transitory combination when the 'fixation' is prevented by ribonuclease. Susceptibility to the inactivating effect of chymotrypsin is not, therefore, caused by any major change of the phage particle, such as separation of its protein from its nucleic acid (Hershey & Chase, 1952). Whether the moment when normally combined phage ceases to be susceptible to chymotrypsin coincides with such a major change is unknown, likewise what susceptibility to chymotrypsin means. Active phage is lost, but this could occur for a variety of reasons ranging from destruction of phage particles to their becoming cemented to bacteria. It is not clear whether the period of combination before 'fixation' is equivalent to the reversible combination described by Garen & Puck (1951) with coli phages. Their phages and hosts were separable only when the ionic content of the medium was made unsuitable for combination, whereas combination of the Rhizobium phage with host in the presence of ribonuclease is reversible in media otherwise favourable for infection.

Table 5. *Effects of ribonuclease and chymotrypsin on combination between phage and bacteria killed by* UV-*radiation*

48 hr. bacterial culture (5×10^7 bacteria/ml.) was irradiated as a layer 0·12 cm. deep in a Petri dish. Each sq.cm. of irradiated area received 0·25 joule of energy of radiation of $\lambda = 2537$ A. The fluid was rocked during irradiation. The proportion of bacteria that remained able to form colonies was 10^{-5}.

0·5 ml. of a phage stock culture diluted 10^{-5} was added to each mixture which was then incubated for 3 hr. at 25°. Samples of the mixtures were then centrifuged for 20 min. at 10,000 r.p.m. and phage was assayed in the supernatant fluids and in the non-centrifuged mixtures.

	Composition of mixtures (in ml.)			Concentration of active phage (plaques/ml.)		
	4 ml. of irradiated bact. culture +				After 3 hr. incubation	
Test no.	1 % ribonuclease solution	0·1 % chymotrypsin solution	Buffer	Initial	Non-centrifuged mixture	Supernatant fluid of a centrifuged sample
1	—	—	1·0	1600	120	100
2	0·5	—	0·5		1300	1200
3	—	0·5	0·5		120	115
4	0·5	0·5	—		350	340

Effects of ribonuclease and chymotrypsin on the interaction between phage and bacteria killed by ultraviolet radiation

Bacteria killed by ultraviolet (UV) radiation can still combine specifically with phage, but how far the normal stages of infection proceed is unknown. Tests were therefore made to compare the effects of ribonuclease and chymotrypsin on the combination of phage with UV-killed bacteria and live bacteria. Tests were also made to see whether phage combined with UV-killed bacteria could later be separated by ribonuclease.

Table 5 shows that the concentration of active phage decreased in the presence of the killed bacteria and remained unaffected when the bacteria were centrifuged out (test no. 1, Table 5). Evidently the phage that was combined with the killed bacteria could not infect live bacteria. The presence of ribonuclease prevented permanent combination of phage with the killed bacteria (test no. 2). Chymotrypsin alone had no effect on the combination (test no. 3), but when present together with ribonuclease, it counteracted the effect of the latter so that the concentration of active phage decreased at a rate only slightly smaller than in the absence of ribonuclease (test no. 4). Separate and joint effects of the two enzymes on combination of phage with UV-killed bacteria were the same as with live bacteria, so it seems reasonable to conclude that the mechanism of interaction of phage with UV-killed bacteria is identical with that of early stages of its interaction with live bacteria.

Addition of ribonuclease to mixtures of phage + killed bacteria which had been incubated together for 3 hr. at 25°, did not liberate any active phage, but it prevented the combination of any further phage. The inability of ribonuclease to liberate 'fixed' phage suggests that the interaction between phage and UV-killed bacteria may go beyond the stage of 'fixation'.

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The Utilization of Acetate by Wild-type and Mutant *Chlamydomonas dysosmos*

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SUMMARY: *Chlamydomonas dysosmos* is capable of normal photosynthetic growth, and of heterotrophic growth in darkness on acetate, pyruvate or lactate. An ultra-violet-induced mutant (D. 2075) was isolated which behaved like an obligate photo-autotroph. In the presence of 2:4-dinitrophenol (5×10^{-5} M) or sodium azide (1×10^{-5} M), the wild-type organism similarly behaved in growth experiments like an obligate photo-autotroph. Mutant cells oxidized all added acetate to CO_2. Wild-type cells oxidized a portion of the substrate, and utilized some of the energy so released for the assimilation of the remainder. There are indications that in the wild type the assimilation of acetate commences only after a period of acetate oxidation. The mutant is believed to be impaired in its ability to effect oxidative assimilation.

The genus *Chlamydomonas* comprises several hundred species of unicellular green flagellates, all capable of growth by fixing atmospheric carbon dioxide in light. For many of these species photosynthesis apparently constitutes the only possible mode of nutrition, since they are unable to grow heterotrophically on organic sources of carbon. Lewin (1950) investigated obligate autotrophy in *C. moewusii* Gerloff without reaching any conclusions as to its underlying causes. It seemed that a fresh approach might be made to the problem by starting with a facultatively heterotrophic species and obtaining a mutant strain which lacked the ability to grow in darkness. Ten distinct species in pure culture were tested for ability to grow in darkness on a variety of carbon sources; of these two appeared capable of heterotrophy. They were identified as *C. dysosmos* Moewus (homothallic) and *C. debaryana* Goroschankin (heterothallic). Both grew sufficiently rapidly in light in simple mineral media with a generation time of 10–12 hr., and neither had specific organic growth-substance requirements. Since *C. dysosmos* grew more rapidly than the other species when supplied with acetate in darkness, it was selected for further study.

MATERIALS AND METHODS

The strain used, originally isolated from a sample collected in Long Island by Mr I. C. G. Cooper (see Cooper, 1952) has been tentatively identified as *Chlamydomonas dysosmos*. Unfortunately it has not been possible to compare characters with the original culture described by Moewus (1931), since the latter was never isolated in pure culture (Moewus, personal communication).

The basal medium (Lewin, 1951) was modified as described below, and solidified with agar (1 %; w/v) for surface cultures. Cells were grown in sterile conditions under constant light and temperature (23°) unless otherwise stated.

For respirometric studies two modes of cultivation and starvation were found satisfactory:

(*a*) *Liquid cultures* (1·0 l.) under constant aeration were grown for 10 days in light (450 f.c.), then aerated for 3 days in darkness in order to deplete endogenous reserves. Cells were separated by centrifugation, washed once, and resuspended in phosphate buffer.

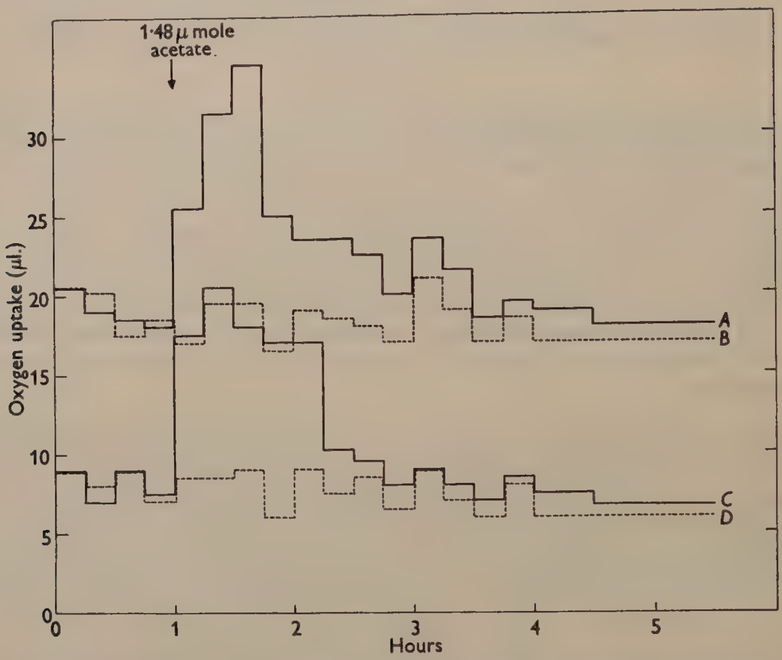

Fig. 1. Effect of composition of growth medium on acetate oxidation by *Chlamydomonas dysosmos* wild-type cells. *A*, cells grown on mineral agar, with nitrate as sole source of nitrogen, 1·48 μmole acetate added after 1 hr.; *B*, as *A*, buffer added after 1 hr.; *C*, cells grown on mineral agar + 0·2 % NH_4Cl, 1·48 μmole acetate added after 1 hr.; *D*, as *C*, buffer added after 1 hr. Cell suspensions all of same optical density.

(*b*) *Surface cultures* on agar medium in Petri plates were grown for 7 days in light (220 f.c.), then incubated overnight in darkness; this promoted motility and ensured a more uniform suspension. Cells were washed off in buffer and used without further treatment. It was found empirically that the incorporation of an ammonium salt (e.g. 4×10^{-3} M-NH_4Cl) markedly reduced post-dissimilatory stimulation (see p. 465 and Fig. 1), and for this reason the basal medium was modified accordingly.

Certain physiological differences were observed between the behaviour of cells grown in these two ways (cf. Winzler, 1940). The second method was preferable, since a starvation period was found not to be essential and results were more closely reproducible; it was employed in all experiments reported in this paper.

Mutant D.2075 was obtained by surface irradiation (Lewin, 1952) with ultraviolet radiation from a General Electric 'Disinfectaire' 38 W. tube. Wild-type cells from a young culture were spread on an agar medium supplemented with sodium acetate ($7\cdot5\times10^{-3}$M), irradiated at a distance of 20 cm. from the ultraviolet source for 10 sec., and then incubated in darkness for 14 days. Cells capable of heterotrophic growth formed colonies, the positions of which were marked with ink; the plates were then illuminated for 4 days to permit growth by photosynthesis of non-heterotrophs. Cultures from this second crop of colonies were tested for ability to grow in darkness on acetate, and among those which lacked this capacity mutant D.2075 was selected for further study. In other respects cells of this mutant strain appeared normal.

Oxygen taken up in respiration was determined manometrically at 25° using a circular Warburg apparatus (manufactured by Braun, Melsungen, Germany). Each vessel contained 30–100 μl. packed cells (centrifuged to constant volume) suspended in 2·0 ml. phosphate buffer (0·1 M-KH_2PO_4, pH 5·6), with 0·2 ml. 10 % KOH in the centre well. Flasks were shaken at 120 oscillations/min., with an amplitude of 3 cm. After allowing 15 min. for equilibration and about 1 hr. to establish endogenous respiration, the substrate, as a sodium salt dissolved in 0·5 ml. of phosphate buffer, was tipped in from a side-arm. In determinations of respiratory quotients (Umbreit, Burris & Stauffer, 1949), alkali was replaced by water in duplicate flasks.

Cell extracts were prepared by the use of a pressure chamber (Milner, Lawrence & French, 1950). A dense suspension was compressed to 5000 lb./sq.in. and released through a controlled nozzle to atmospheric pressure. The resulting homogenate contained few if any intact cells; it was freed from cell debris by centrifugation.

RESULTS

Growth experiments

The ability of wild-type cells to grow in darkness was tested on solid media supplemented with ten different carbon sources and adjusted to pH 6·1. Each source was tested at three different concentrations, with nitrogen in the form of nitrate, ammonia, or Bacto-Tryptone (Difco). Heterotrophic growth was good in media supplemented with acetate, slight with lactate or pyruvate, and absent with succinate, malate, citrate, glutamate, glycerol, glucose or phosphoglycerate, at concentrations which were not inhibitory in light. Apart from indirect effects on the pH value of the medium, the sources of nitrogen did not alter these results.

The mutant D.2075 was virtually unable to grow on acetate, lactate or pyruvate in the absence of light. The addition of various concentrations of yeast extract did not restore its ability to utilize acetate for growth, indicating that the mutant was probably not a vitamin-deficient one.

Manometric studies of respiration

General observations. In an attempt to elucidate the physiological basis for the mutant character, the effects of the addition of substrates or respiratory inhibitors upon respiration of mutant and wild-type cells were investigated manometrically.

Cells which had been grown in liquid culture, and had not been subjected to a period of starvation after growth in light, showed no detectable stimulation of oxygen uptake on the addition of any substrate. However, after aeration for 3 days in darkness, the endogenous rate was decreased, and stimulation of respiration by acetate was observed.

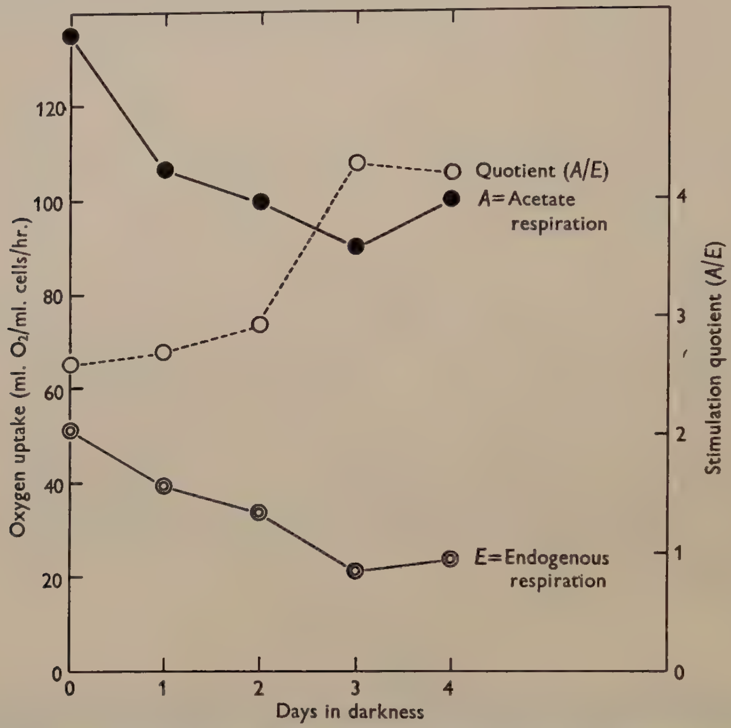

Fig. 2. Effect of incubation in darkness on respiratory rate of *Chlamydomonas dysosmos* wild-type cells, previously grown in light for 7 days.

Cells grown on solid media showed stimulation by acetate even when not subjected to previous starvation in darkness, though relative stimulation was more marked after such treatment (Fig. 2). Such cells, incubated in darkness overnight, had an endogenous respiratory rate equivalent to 0·5–0·8 ml. oxygen/ml. cells (packed wet volume)/hr. corresponding to about 50–80 ml. O_2/g. cell-N/hr. This rate was approximately doubled, both in wild-type and in mutant cells, on the addition of acetate (Figs. 3, 4). The total volume of oxygen taken up, after correction for endogenous respiration, was proportional to the acetate added within the limits 2×10^{-4}M to 1×10^{-3}M. At higher concentrations inhibition effects precluded quantitative determinations. Measurable differences were observed after the addition of quantities as small as 25 μg. acetic acid per flask. No appreciable stimulation of oxygen uptake occurred after the addition of pyruvate or lactate at any concentration tested.

Stoichiometry of acetate oxidation. The total volume of oxygen uptake during the dissimilation of acetate by cells of the mutant D. 2075 was chemically

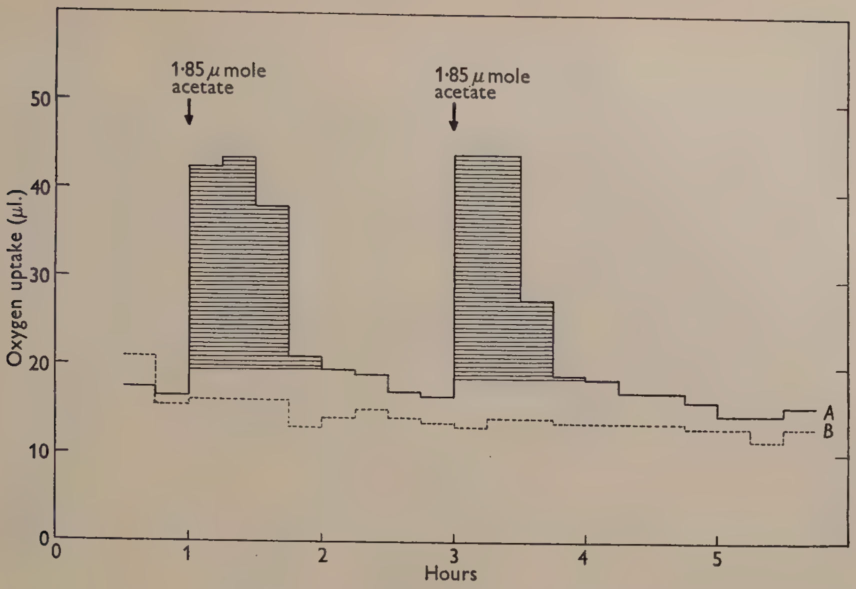

Fig. 3. Effect of two consecutive doses of acetate on respiration of *Chlamydomonas dysosmos* wild-type cells. *A*, 1·85 μmole acetate added after 1 hr. and after 3 hr.; *B*, buffer added after 1 hr. and after 3 hr.

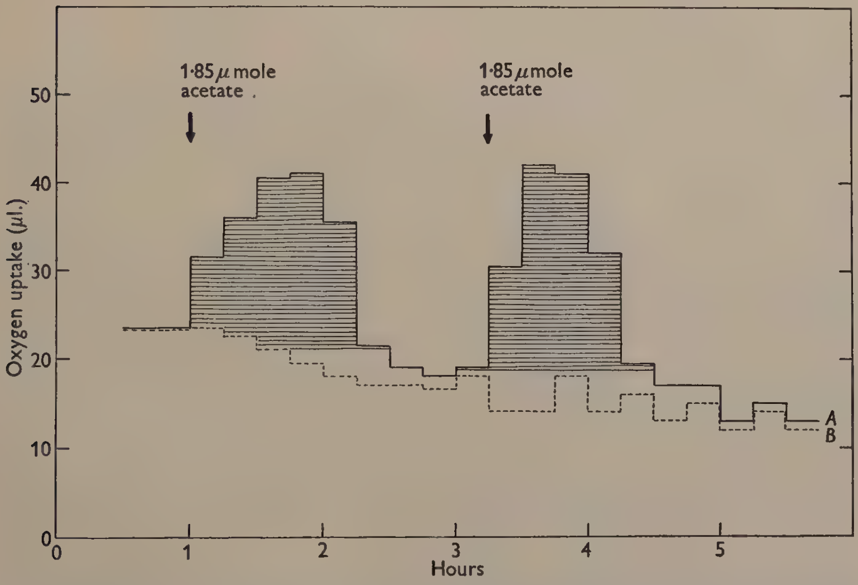

Fig. 4. Effect of two consecutive doses of acetate on respiration of cells of *Chlamydomonas dysosmos* mutant D.2075. *A*, 1·85 μmole/acetate added after 1 hr. and after 3·25 hr.; *B*, buffer added after 1 hr. and after 3·25 hr.

equivalent to complete oxidation of the acetate supplied, according to the equation:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O. \quad (1)$$

The molar ratio of O_2/acetate was 2·0, indicating that no acetate was left to be assimilated: hence the inability of this strain to grow heterotrophically. On the other hand, when wild-type cells were similarly supplied with acetate, the volume of oxygen taken up was found to be dependent on the number of cells per vessel (compare Figs. 5 and 6). The O_2/acetate ratio was considerably less than 2·0 when high cell densities were not used. This relationship was unexpected, but could be accounted for on the following postulates: all cells possess a mechanism for oxidizing acetate quantitatively to CO_2, and this is the only system operative in the mutant D. 2075; in wild-type cells, however, a second mechanism comes into play within an hour or so of contact with acetate, enabling this substrate to be usefully assimilated for anabolism. When the ratio of cells to acetate is high, the acetate is all oxidized before the anabolic mechanism becomes operative; when there are fewer cells, acetate disappears from the medium more slowly, and some remains long enough to permit assimilation via the second system. This hypothesis was tested by measuring the oxygen uptake during the utilization of two equal doses of acetate, added successively to the same sample of wild-type cells. It was found that the O_2/acetate ratio was lower (1·3) for the second dose than for the first (1·7), indicating that the assimilatory mechanism had, in fact, become more effective as a result of the first period of acetate utilization, and adding support to the suggestion that the assimilatory system takes some time to develop. A similar experiment, carried out with mutant cells, gave a ratio of 2·0 for each dose. It was observed that, both in wild type and mutant, the second dose was utilized rather more rapidly than the first.

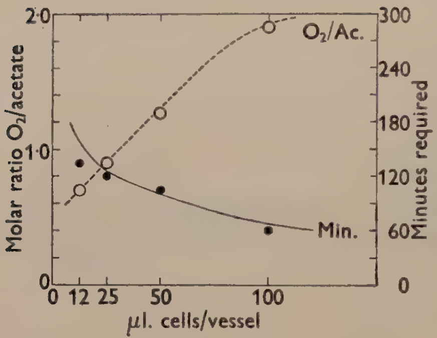

Fig. 5

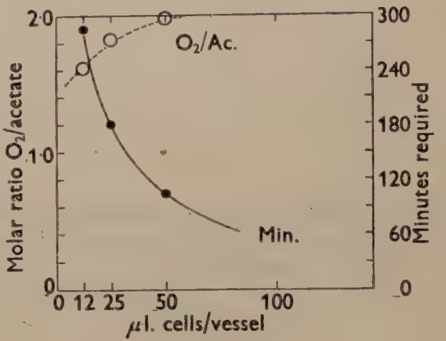

Fig. 6

Fig. 5. Effect of cell density on oxygen uptake (mole oxygen/mole acetate) and time required for acetate utilization in *Chlamydomonas dysosmos* wild-type cells.

Fig. 6. Effect of cell density on oxygen uptake (mole oxygen/mole acetate) and time required for acetate utilization in cells of *Chlamydomonas dysosmos* mutant D. 2075.

Respiration following completion of acetate oxidation. The interpretation of the Warburg data tended to be complicated by changes in the concurrent endogenous respiration. On the addition of acetate, oxygen uptake rose rapidly to a higher rate, remained steady for a period, and then fell rapidly as the substrate was used up, However, in both wild type and mutant, the respiratory rate after acetate dissimilation did not return at once to the original level, but for *c.* 1 hr. tended to remain higher than that in parallel control flasks to which only buffer had been added (see Figs. 3, 4). Since the complete oxidation of the added substrate by mutant D. 2075, according to equation (1) above, could only just account for the stimulated respiration indicated by the shaded area in the oxygen-uptake curve, some explanation was needed for the subsequent increased rate of respiration. Apparently in *Chlamydomonas dysosmos*, under the given conditions, endogenous respiration not only continued unsuppressed while cells were oxidizing an externally supplied substrate, but was actually stimulated thereby, proceeding at an augmented rate for an hour or more after the acetate had been consumed. Such an effect might be attributable to an increased activity of enzymes involved in terminal oxidation, or to an increase in the supply of intermediates in the cyclic processes concerned. Inspection of curves published by various workers suggests that this phenomenon is not uncommon—cf. studies on the oxidation of glucose by *Escherichia coli* (Cook & Stephenson, 1928) and by *Scenedesmus quadricauda* (Taylor, 1950).

Respiratory quotients. In both wild-type and mutant cells, the respiratory quotient rose from 0·9 during endogenous respiration to 1·0 during acetate dissimilation. After the exhaustion of the substrate, as indicated by an abrupt drop in the respiratory rate, the R.Q. of the mutant returned at once to 0·9, but that of wild type remained about unity for *c.* 1 hr. before falling to the original level (see Table 1). This may indicate that after acetate respiration in normal cells there remain intermediates which tend for a while to accelerate the oxidation of endogenous carbohydrate.

Table 1. *Respiratory quotients of cells of* Chlamydomonas dysosmos *wild type and mutant D.* 2075 *before, during, and after acetate oxidation*

	Endogenous	During acetate oxidation	0–1 hr. after acetate oxidation	1–4 hr. after acetate oxidation
Wild-type cells				
Number of 1 hr. determinations	16	6	6	6
Respiratory quotient; average and standard deviation	0·93 ± 0·055	1·00 ± 0·033	0·98 ± 0·020	0·90 ± 0·030
Mutant D. 2075 cells				
Number of 1 hr. determinations	6	4	4	9
Respiratory quotient; average and standard deviation	0·88 ± 0·055	0·99 ± 0·009	0·90 ± 0·044	0·87 ± 0·041

Comparison of Chlamydomonas dysosmos *with other species.* Since the original objective had been to attempt to determine the underlying causes of obligate autotrophy, a comparison was made between *C. dysosmos*, which is normally capable of growth in darkness on acetate media, and *C. moewusii*, which is not. Though it was optimistically expected that *C. moewusii* would behave like the mutant D. 2075 of *C. dysosmos*, which oxidizes all of the added acetate to CO_2, this was found not to be the case. In *C. moewusii*, like wild-type *C. dysosmos*, the R.Q. for acetate oxidation was 1·00, but the molar ratio O_2/acetate was only 1·0–1·5, and did not rise with increasing cell density. The reason for the failure of this species to utilize acetate for growth remains obscure.

In *Chlamydomonas debaryana* the O_2/acetate ratio was likewise about 1·0–1·5, and was apparently unaffected by different cell densities over the range tested. A mutant of this species was obtained by the method described on p. 460. It resembled *C. dysosmos* D. 2075 in being unable to grow on acetate media, but showed no respiratory stimulation whatsoever when supplied with acetate. It is evident that in this case the inability to grow heterotrophically must be attributed to an impairment completely different from that of *C. dysosmos* D. 2075 on the one hand and *C. moewusii* on the other.

Effects of various agents on acetate utilization

Nitrogen sources. The character of the nitrogen source, whether nitrate or an ammonium salt, did not appreciably affect the ability of the wild type or the inability of the mutant to grow heterotrophically on acetate media. Endogenous respiration and acetate oxidation were unaffected by the addition of nitrate (0·01 M-KNO_3) or ammonia (0·01 M-NH_4Cl) to the phosphate buffer in which the cells were suspended.

Dinitrophenol. The effect of 2:4-dinitrophenol (DNP) on many oxidation systems is apparently to uncouple phosphorylation from oxidation, so that the latter process proceeds with wasteful dissipation of all the chemical energy released, and assimilation is thereby prevented (Simon, 1953). It was found that DNP (5×10^{-5} M) or azide (1×10^{-5} M), added with acetate in agar media at pH 6·1, permitted apparently normal growth of wild-type *Chlamydomonas dysosmos* in light, but completely inhibited heterotrophic growth in darkness (see Table 2). In this way the effect of the D. 2075 mutation was simulated by the action of an inhibitor on wild-type cells. (The fact that 1×10^{-4} M-DNP

Table 2. *Effects of 2:4-dinitrophenol and of azide on growth of* Chlamydomonas dysosmos *wild type and mutant D.* 2075

Inhibitor	Concentration ($\times 10^{-5}$ M)	pH	Wild type		Mutant D. 2075	
			Light	Darkness	Light	Darkness
None	—	6·9	+	+	+	−
2:4-Dinitrophenol	2·5	6·7	+	+	+	−
	5·0	6·5	+	−	+	−
	10·0	6·1	+	−	−	−
	20·0	5·7	−	−	−	−
Azide	0·4	6·9	+	+	+	−
	0·8–3·2	6·9	+	−	+	−
	6·4	6·9	−	−	−	−

Basal medium: minerals + $7·5 \times 10^{-3}$ M-acetate.
Growth period: 14 days. + = growth.

inhibited photosynthetic growth of the mutant, but not of the wild type, is not readily explained.)

Attempts to demonstrate respirometrically the uncoupling of oxidative assimilation by DNP (cf. Clifton, 1946; Winzler, 1940) were complicated by the high rate of endogenous respiration in this organism. 1×10^{-6} M-dinitrophenol stimulated endogenous respiration by about 20 %, without significantly affecting the rate or extent of acetate oxidation. 5×10^{-6} M or 1×10^{-5} M-DNP (pH 5·6) caused the rate of endogenous respiration, as measured by oxygen uptake, almost to double (Figs. 7, 8). The addition of acetate to cells so treated caused the rate of oxygen uptake to rise still further for a period. However, since it could not be established that endogenous respiration continued unchanged during this period, the oxygen consumption attributable to acetate dissimilation (i.e. the O_2/acetate ratio) could not be determined. A still higher concentration of DNP, $2{\cdot}5\times10^{-5}$ M, decreased endogenous respiration to about a quarter of the control rate, and cells so treated showed no stimulation of oxygen uptake on the addition of acetate.

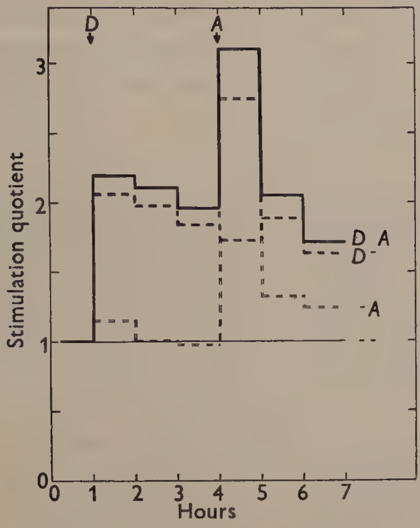

Fig. 7

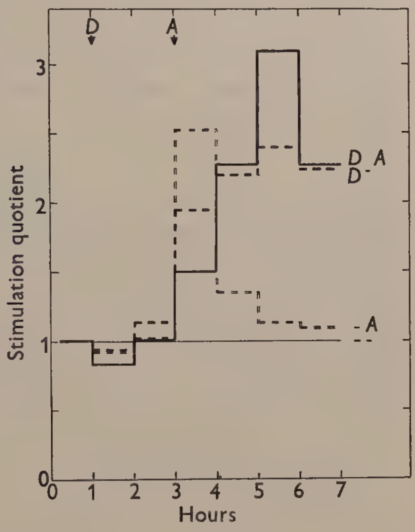

Fig. 8

Fig. 7. Effect of the addition of 2:4-dinitrophenol (DNP) (5×10^{-6} M) on relative respiratory rate of *Chlamydomonas dysosmos* wild-type cells. (*D*-) and (*DA*), DNP added after 1 hr.; (–*A*) and (*DA*), acetate (1·85 μmole) added after 4 hr. All rates calculated as ratios of control flasks, to which only buffer was added after 1 hr. and after 4 hr.

Fig. 8. Effect of the addition of 2:4-dinitrophenol (1×10^{-5} M) on relative respiratory rate of *Chlamydomonas dysosmos* wild-type cells. Key: see Fig. 7.

Cell extracts. To test the possibility that cells of the mutant D.2075 might produce some chemical agent which acted in the same way as DNP (cf. Millerd, Bonner & Biale, 1953), a cell-free extract of mutant D.2075 was prepared. This extract by itself showed no oxygen uptake, before or after the addition of acetate. In wild-type cells, suspended in extract of mutant and then permitted to oxidize acetate, the O_2/acetate ratio remained unchanged. A reciprocal experiment, in which mutant D. 2075 cells were treated with wild-type extract, also gave a negative result. Thus in neither case was it possible to demonstrate the presence of a soluble factor, capable of entering intact cells, which might be responsible for the physiological difference between mutant and wild type.

Glycollate and succinate. Anderson (1945) found that, in the presence of glycollic acid, acetate oxidation by *Prototheca zopfii* resulted in an oxygen uptake in excess of that required for complete dissimilation of the latter substrate; and Goldschmidt & Powelson (1953) observed a marked stimulation of acetate dissimilation by *Staphylococcus aureus* in the presence of succinate. Glycollate ($2 \cdot 5 \times 10^{-3}$ M) and succinate ($7 \cdot 5 \times 10^{-4}$ M) were therefore tested on acetate dissimilation by *Chlamydomonas dysosmos*. No stimulation of respiratory rate was observed in either case, nor was the O_2/acetate ratio altered in the presence of these acids.

DISCUSSION

It appears that the mutant D. 2075 of *Chlamydomonas dysosmos* is unable to grow in darkness heterotrophically because it can only wastefully consume the supplied substrate. Either it lacks the ability to condense acetate in an anabolic mechanism, or it is unable to couple the energy of acetate oxidation in some synthetic pathway. One is reminded of other examples of organisms capable of oxidizing certain organic compounds which nevertheless do not serve as substrates for growth; some of these are summarized in Table 3.

Table 3. *Examples of organisms capable of oxidizing a substrate not utilized for growth*

Organism	Substrate	Observations	Reference
Acetobacter suboxydans	Lactate, etc.	Blocked beyond acetate	King & Cheldelin (1953)
Azotobacter agilis A. 3	Succinate, etc.	Blocked beyond pyruvate	Karlsson (1950)
Escherichia coli	Formate	100 % oxidized	Cook & Stephenson (1928)
Sphaerotilus natans	Benzoate	48 % oxidized	Stokes (1954)
Chlamydomonas moewusii	Acetate, etc.	70 % oxidized	Lewin (1950)
Navicula pelliculosa	Lactate, etc.	R.Q. = 0·96 (lactate)	Lewin (1953)
Aspergillus oryzae	Formate, acetate, etc.	0·5 M, pH 5·5	Tamiya (1932)

On the other hand, in light on acetate media in an atmosphere free from CO_2, growth was as rapid in the mutant as in wild-type cultures. It may be that the cells were oxidizing acetate, and that a proportion of the CO_2 so formed was at once fixed intracellularly by normal photosynthetic pathways. However, in the experiments of Allison, Skipper, Reid, Short & Hogan (1953) it was found that though assimilation of acetate by *Nostoc muscorum* was considerably accelerated by concomitant photosynthesis, this process was not mediated through CO_2 as an intermediate. The possibility remains that cells of *Chlamydomonas dysosmos* mutant D. 2075, though unable to employ the energy from acetate oxidation for acetate assimilation, could nevertheless use light energy for this purpose.

In wild-type *Chlamydomonas dysosmos*, though some acetate can be assimilated, the respiratory quotient during acetate oxidation is unity, indicating that under the experimental conditions the cells are not synthesizing appreciable quantities of fat. This seems to conform with the pattern of acetate utilization in *Scenedesmus*, as reported briefly by Calvin *et al.* (1951). These workers supplied unstarved cells with labelled acetate ($^{14}CH_3.COOH$) in the presence of 4 % CO_2. They found that after 30 min. in darkness the labelling appeared in organic acids, chiefly citric and glutamic, indicating the operation of a tricarboxylic acid cycle; in bright light, on the other hand, the acetate was largely assimilated into lipids. Obviously the use of tracers is indicated if more critical studies of *Chlamydomonas* metabolism are to be carried out, specifically in relation to the products of acetate assimilation.

A value of 1·5 for the ratio of O_2/acetate was obtained for acetate respiration in *Escherichia coli* by Cook & Stephenson (1928) and in *Mycobacterium ranae* by Lindsay, O'Donnell & Edson (1950), who in each case compensated for simultaneous endogenous respiration. Santer & Ajl (1954) clearly demonstrated that in *Pasteurella pestis* such compensation was justifiable, since the rate of endogenous respiration remained unchanged during acetate oxidation. van Niel (1941) obtained a ratio of 1·0 for acetate respiration in *Spirillum rubrum*, with cells which had been starved to a degree where endogenous respiration was negligible, so that in this case it was not necessary to correct for oxygen uptake in controls. On the other hand, in studies of algae by Barker (1935) with *Prototheca zopfii*, and by Myers (1947) with *Chlorella pyrenoidosa*, O_2/acetate ratios of about 1·0 were calculated on the assumption that endogenous respiration was suppressed during the period of acetate oxidation. This cannot be true for *Chlamydomonas dysosmos* for the following reason. In the mutant D. 2075, or in dense suspensions of wild-type cells, the addition of acetate causes a net increase in oxygen uptake equivalent to 2 mole O_2/mole substrate—i.e. the latter is completely oxidized. If one were to assume that endogenous respiration is suppressed during acetate oxidation, one would calculate an O_2/acetate ratio far exceeding 2·0, which is chemically impossible.

Under the action of certain agents, assimilatory processes can be dissociated from respiration in many organisms. Thus Clifton (1946) observed that, in the presence of suitable concentrations of DNP, assimilation could be dissociated from respiration so that the oxidation of substrates went virtually to completion. Concentrations of DNP or azide which had a negligible effect on the rate of endogenous respiration in *Escherichia coli* or *Pseudomonas calco-acetica* raised the O_2/acetate ratio from 1·5 to 1·9. Winzler (1940) obtained similar results using *Saccharomyces cerevisiae*, in which the O_2/acetate ratio was raised from 1·3 to 2·0. Attempts to demonstrate such an effect in *Chlamydomonas dysosmos* were complicated by the high rate of endogenous respiration in this organism (see p. 467). Nevertheless, the fact that 5×10^{-5}M-dinitrophenol inhibited heterotrophic growth on acetate, while permitting photosynthetic growth, suggests that here, too, oxidative assimilation may be inhibited by this agent.

Millerd *et al.* (1953) presented evidence for the presence in ripe avocado pears of an agent which acts in a fashion similar to DNP. Cell-free preparations of pre-climacteric fruits were capable of coupling substrate oxidation with esterification of inorganic phosphate, whereas similar preparations of post-climacteric fruits were unable to form high-energy phosphate compounds in this way. The difference in behaviour was traced to a soluble factor present in the post-climacteric fruits. No factor of this sort could be detected in cells of the *Chlamydomonas dysosmos* mutant D. 2075.

Finally, it seems that in *Chlamydomonas dysosmos* there are two systems for dealing with acetate, one dissimilatory and the other assimilatory. The second system is only operative from wild-type cells after a lag, and is apparently absent altogether from the mutant D. 2075. Barron & Ghiretti (1953) adduced evidence indicating that acetate oxidation in various organisms may take place either through the Krebs tricarboxylic acid cycle, or by an alternative pathway which involves dicarboxylic acids. Krebs, Gurin & Eggleston (1952) suggested that in yeast the latter system may mediate the oxidative dissimilation of the substrate, whereas the tricarboxylic acid cycle is responsible for the provision of metabolic intermediates. If in fact *C. dysosmos* normally possesses two systems of this sort, it is possible that the mutant character of D. 2075 lies in some impairment of the Krebs cycle. Cultures of *C. dysosmos*, wild type and mutant D. 2075, have been deposited with the algal type culture collections at Cambridge, England, and at Bloomington, Indiana, U.S.A.

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Some Facultatively Anaerobic Gram-negative Rods from the Rumen of the Calf and the Sheep

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SUMMARY: The coliform population in the rumens of twelve calves of ages 10–105 days was never greater than 10^6/g., and consisted mostly of *Escherichia coli* and intermediate types. Only one well-capsulated strain of *Aerobacter aerogenes* was isolated, whereas such capsulated strains could be isolated with ease from the rumen of a starch-fed sheep. An unidentified small catalase-negative Gram-negative rod, which readily produced ammonia from urea, was isolated from the rumen contents of two of the older calves, at a dilution of 10^{-4}.

Mann, Masson & Oxford (1954*a*) showed that although the coliform population of the adult hay-fed sheep's rumen was numerically quite small, nevertheless it consisted mostly of *Escherichia coli* of intestinal type rather than *Aerobacter aerogenes* or *A. cloacae,* the common saprophytes found on plants and grains. *A. aerogenes* in the capsulated form has, however, since been isolated quite easily from the rumen of a starch- and concentrate-fed sheep (see below). It seemed of some interest therefore to study the distribution of coliform types in the rumen of the young ruminant, particularly the calf when reared by the usual method on milk and a 'starter' gruel, from the first tentative nibbling of straw at age 1–2 weeks to the establishment of the complete rumen microflora after discontinuance of milk in the ration. This has been now carried out in conjunction with the aureomycin feeding of calves described by Mann, Masson & Oxford (1954*b*) which resulted in the isolation of lactobacilli from the rumens of the younger calves only (reported by Mann & Oxford, 1954*a*). A description is now given of certain other unidentified non-coliform Gram-negative rods, also isolated from the calf rumen in the same experiment. These appeared to be of possible importance in the functioning of the rumen microflora because of their greater ability to produce ammonia from urea than is possessed by *A. aerogenes* or *A. cloacae.*

METHODS

Feeding of calves. This is fully described by Mann *et al.* (1954*b*). The calves (twelve in all) were fed 'starter' gruel by bucket. Hay and grass were available after the 3rd week, but fresh milk was discontinued after the 6th week. The three youngest calves examined (10–11 days old; group OC in Table 1) were slaughtered during March–May 1954 and were therefore separate from the main aureomycin-feeding experiment of May–August 1953.

Isolation of rumen Gram-negative rods in pure culture. The bottle-counting technique used for sheep rumen contents by Heald, Krogh, Mann, Appleby, Masson & Oxford (1953) and for the isolation of rumen lactobacilli by Mann & Oxford (1954*a*), although suitable for saccharolytic Gram-positive bacteria, cannot be relied upon to give a true representation of rumen saccharolytic Gram-negative bacteria when these are in the minority. It was therefore replaced by the following method. A sample of rumen contents (10 g.) taken from the well-mixed total contents of a rumen as soon as possible after slaughter of the calf (i.e. within 2 hr.) was placed in a sterile McCartney bottle, diluted with an equal weight of sterile saline, gassed with sterile CO_2 and shaken mechanically for 20 min. to detach as many bacteria as possible from plant particles. The mixture was then centrifuged very lightly to bring down the larger plant particles, and serial ten-fold dilutions made from the supernatant in sterile saline. One ml. from each dilution was transferred to a sterile Petri dish, and incorporated at 45° in 15 ml. of Wright's nutrient agar containing glucose (0·2 %) and crystal violet (0·0005 %). This low concentration of crystal violet did not inhibit all Gram-positive bacteria, but did allow the maximum number of Gram-negative bacteria (coliforms and otherwise) to form distinct colonies in 48 hr. when the plates were incubated anaerobically at 38°. All the Gram-negative isolates obtained in this study proved however to be facultative anaerobes. They were purified by repeated plating on glucose + peptone + yeast extract + salt agar and maintained till required in Robertson's cooked meat medium.

Examination of Gram-negative rod isolates. Those which fermented lactose in MacConkey's bile salt medium with production of acid and gas and were also catalase-positive, were further studied by standard methods for coliform bacteria. Indole was tested for with Kovács' reagent, and both O'Meara's and Barritt's modifications of the Voges–Proskauer test were employed. Production of ammonia from urea was tested for by Christensen's (1946) method and also in the more highly buffered medium of Stuart, van Stratum & Rustigian (1945).

Table 1. *Classification of calf rumen coliform bacteria*

Calves: A or A^1, fed aureomycin; C, control not fed aureomycin (see Mann *et al.* 1954*b*). N.B. *A. aerogenes* type II and *A. cloacae* were never isolated.

Calf reference number	Age of calf (days)	No. of isolates of				Total possible intestinal (VP − MR +) types	*A. aerogenes* type I (VP + MR −)
		Escherichia coli		Int.* I	Int. II		
		type I	type II				
OC1	11	2	0	0	0	2	0
OC2	11	1	0	0	0	1	0
OC3	10	0	0	0	0	0	0
1A	33	0	0	0	0	0	0
1A^1	43						
1C	32						
2A	54	0	6	4	0	10	2
2A^1	59	0	0	0	0	0	0
2C	60	2	1	0	0	3	0
3A	105	4	5	0	0	9	0
3A^1	96	0	0	0	0	0	1
3C	86	2	7	0	0	9	2
Total no. of isolates:		11	19	4	0	34	5

* Int. = intermediate coliform types (MR +, VP −, citrate +).

RESULTS

Classification of coliform bacteria isolated from the rumens of calves of various ages from 10 *to* 105 *days*

The viable coliform population was usually 10^4–10^6/g. rumen contents; sometimes much less (10^2/g. in calf 3A), sometimes apparently nil, e.g. in calves 1A, 1A[1], 1C and 2A[1]. The thirty-nine coliform isolates are classified in Table 1 according to the scheme used by Wilson *et al.* (1935, p. 156) for milk coliform bacteria. It will be seen from the table that *Escherichia coli* I and II and the intermediate types, all VP-negative and MR-positive, accounted for 87 % of the total isolates, whilst *Aerobacter aerogenes* (VP + MR −) accounted for only 13 % and *A. cloacae* was not found. All the *A. aerogenes* isolates attacked urea weakly and not consistently. Only one such isolate (out of five) showed well-marked capsulation, and, unlike the other four isolates, fermented adonitol and inositol.

Capsulated strains of Aerobacter aerogenes *from the rumen of a concentrate-fed sheep*

The rumen-fistulated sheep in question was fed the following daily ration: hay (300 g.), concentrates (500 g.), and potato starch (200 g.). The medium used for isolation of coliforms from rumen contents was MacConkey's agar. Capsulated isolates of *Aerobacter aerogenes* could invariably be obtained with ease from the rumen contents of the above sheep, usually at a dilution of 10^{-2} to 10^{-4}. Control experiments with rumen contents from two fistulated hay-fed sheep confirmed the conclusion of Mann *et al.* (1954*a*) that *A. aerogenes* could not be isolated from this source. Twenty-three encapsulated isolates of *A. aerogenes* were obtained in all from six separate samples of the starch-fed sheep's rumen contents. All produced markedly mucoid colonies on agar media containing a fermentable carbohydrate, and were indole and methyl red negative, nitrate-reducing, Voges–Proskauer, citrate, catalase and urease positive. No isolate liquefied gelatin, two only fermented starch, six fermented neither dulcitol nor adonitol, two (the starch-fermenters) attacked adonitol but not dulcitol, one attacked dulcitol but not adonitol, and the remaining fourteen fermented both these sugar alcohols.

Other urease-positive Gram-negative isolates from calf rumen

Of eleven catalase-negative non-coliform isolates, two from the rumen of calf OC1 attacked urea only weakly, whereas the remaining nine from much older calves (five from calf 2C and four from calf 3A[1]) gave the typical reaction in Christensen's medium after 18 hr. incubation at 38°. They did not, however, produce the requisite lowering of pH, comparable with an authentic strain of *Proteus vulgaris*, in the more highly buffered medium of Stuart *et al.* (1945); but the growth was very poor in this instance. All these nine non-coliform urease-positive isolates seemed to be very similar and a short description is appended.

Morphology. Films made from growth on Wright's nutrient agar showed small rods or cocco-bacilli, 0·5–1 μ. × 0·3–0·5 μ., with roughly parallel sides and rounded ends, sometimes in short chains but more often arranged in bundles, difficult to distinguish from aggregations of cocci; Gram-negative, non-motile, non-sporing.

Growth characteristics. The 24 hr. growth on agar consisted of almost colourless, smooth, transparent, punctiform colonies up to 0·8 mm. diameter with entire edge and convex elevation. After 7 days the colonies were still small (2 mm. diameter), pale brown in colour and differentiated into a low convex central zone and an effuse peripheral zone. There was no haemolysis on horse blood agar plates. In nutrient broth, after 48 hr., there was moderate uniform turbidity with granular deposit and thin granular pellicle. The growth range seemed to be from pH 6 to 9. The optimum growth temperature was 38–40°; growth was poor at 50° and at 30°, but still took place at 22°.

Biochemical reactions. The following compounds were fermented with production of acid and gas: arabinose, dextrin, galactose, glucose, glycerol, lactose, maltose, raffinose, rhamnose, sucrose, trehalose and xylose; only slight acidity was produced in litmus milk. The following compounds were not fermented: adonitol, aesculin, dulcitol, inositol, inulin, mannitol, salicin, sorbitol and starch. Methyl red-positive; methylene blue reduced; nitrate reduced to nitrite; Voges–Proskauer reaction negative; gelatin not liquefied; catalase not formed; H_2S not produced. Ammonia was readily produced from urea as previously stated. The organism, however, shows little resemblance in other respects to any species of *Proteus*, as will be seen from the above description.

DISCUSSION

It would seem that the rumen coliform bacteria of the milk-and-gruel-fed young calf closely resemble those of the hay-fed adult sheep both with respect to population ($\not> 10^6$/g. rumen contents) and to type, which is intestinal rather than saprophytic. The urea-attacking *Aerobacter aerogenes* or *A. cloacae* were relatively in the minority, although other unidentified non-coliform Gram-negative bacteria with a more pronounced power of attacking urea were isolated from the rumens of two older calves. It has long been known that mixed rumen bacteria will rapidly produce ammonia from urea, even when the fodder does not contain this source of nitrogen (see Pearson & Smith, 1943). This is not surprising when it is remembered that urea is a normal constituent of ruminant saliva. What is surprising is that an association of preponderantly obligate anaerobic bacteria, as the rumen microflora is thought to be, should have this urea-splitting power so highly developed since, by and large, all the bacteria so far reported as attacking urea in pure culture belong to aerobic or facultatively anaerobic genera like *Proteus*, *Aerobacter*, *Bacterium* (Cooke & Keith, 1927), *Micrococcus* (*Staphylococcus*), *Sarcina*, with apparently no representatives so far among anaerobic genera like *Clostridium*, *Bacteroides*, *Veillonella*, *Propionibacterium*, members of which have at various times been isolated from the rumen. Urease-positive micrococci have in fact been isolated in pure culture from sheep rumen contents (Mann *et al.* 1954*a*) and also from the rumens of the calves used in this study (Mann & Oxford, 1954*b*) but their numbers are usually, although not always, quite small. Hence an interest attaches to the isolation of any new urease-positive small Gram-negative rod from the calf rumen. There must clearly be other urease-positive bacteria in the rumen which have hitherto eluded isolation in pure culture.

A further interest attaches to rumen coliform bacteria of the saprophytic type in regard to their capsulation. Hobson & MacPherson (1953) noted that the final sediment obtained when strained sheep rumen contents was centrifuged consisted largely of capsulated Gram-negative bacteria, occurring in clumps in the case of the starch-fed sheep, rather than in relative isolation as with the hay-fed sheep. This suggests that the presence of starch in the ration may alter the 'free' rumen Gram-negative microflora. Although the population of capsulated *Aerobacter aerogenes* in the starch-fed sheep's rumen is quite small and can account for only a minute proportion of the total 'free' Gram-negative micro-flora, it is perhaps significant that this particular capsulated bacillus is apparently absent from the hay-fed sheep's rumen. This is not inconsistent with the findings of Wilson *et al.* (1935, p. 175) who reported that, in Britain at least, *A. aerogenes* can more easily be isolated from grains and feeding cakes than from hay.

We wish to thank our colleague Dr P. N. Hobson for placing the starch-fed sheep at our disposal.

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An Antagonist of Streptomycin and Dihydrostreptomycin produced by *Pseudomonas aeruginosa*

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SUMMARY: A substance produced by *Pseudomonas aeruginosa* (*P. pyocyanea*) which antagonizes the activity of streptomycin and dihydrostreptomycin, and which will itself inhibit bacterial growth in low concentrations, has been isolated in a crystalline form.

The antagonist at 0·01 μg./ml. was capable of antagonizing the action of 1000 times its weight of dihydrostreptomycin against *Bacillus subtilis* or *B. pumilus.* It was much less active against *Escherichia coli.* Growth of *B. subtilis* and *B. pumilus* was inhibited by less than 1 μg. antagonist/ml. under conditions which required a concentration of 6 μg. dihydrostreptomycin sulphate/ml. to inhibit growth.

A method of assay of antagonist activity, capable of assaying solutions containing 0·01 μg. antagonist/ml., was developed. It was a cup-plate method which depended on the production of zones of exhibition. Plates poured with seeded nutrient agar containing an inhibitory concentration of dihydrostreptomycin were used, and the samples of antagonist were placed on the surface of the agar in fish spine beads. The zones of exhibition developed during incubation of the plates and the potency of the samples could be determined from a comparison with zones produced by a standard preparation on the same plate.

The antagonist was produced by most strains of *Pseudomonas aeruginosa* under favourable conditions. Its production in fluid culture depended on vigorous aeration, but too high an aeration rate or foaming of the culture produced low yields. Strains of other organisms which were examined, for example, *Staphylococcus aureus, Escherichia coli, Serratia marcescens, Bacterium bodenheimer*, streptococci of Lancefield's Group D, *Bacillus subtilis* and *B. pumilus*, did not produce antagonist.

The antagonist was extracted from fluid culture by *n*-butanol; the butanol was removed by distillation in an atmosphere of nitrogen, and the residue fractionated by precipitation from benzene solution by addition of light petroleum. The success of the fractionation was dependent on obtaining an initial butanol extract containing at least 100 μg. antagonist/mg. total solids. This in turn was dependent on the use of a simple chemically defined culture medium for production, and the choice of a strain of *Pseudomonas aeruginosa* which produced a high yield of antagonist. The crystalline antagonist has the formula $C_{17-18}H_{23-24}NO_2$ and a molecular weight of the order 300–400. It is stable in the dry crystalline state, but readily oxidized in solution. It is soluble in ethanol and butanol, less soluble in benzene, and relatively insoluble in light petroleum. Its maximum solubility in water at room temperature at pH 7·0 is *c.* 10 μg./ml.; at pH 9·0 it is 20–30 μg./ml.

The work described in this paper developed from a search for an effective means of inactivating streptomycin and dihydrostreptomycin which could be applied in sterility tests of these antibiotics. Streptomycin may be inactivated by carbonyl reagents, and sterility tests based on this principle are used. As these reagents are in themselves bacteriostatic, the tests have not proved very satisfactory. Dihydrostreptomycin differs from streptomycin in having the labile aldehyde group reduced, so that its activity is unaffected by carbonyl reagents. It is a very stable molecule and can only be destroyed by chemical means which also destroy contaminating bacteria. Since substances which inactivate dihydrostreptomycin are almost certain to inactivate streptomycin whilst the reverse does not necessarily obtain, dihydrostreptomycin has been used in most of this investigation.

Fitzgerald & Bernheim (1948) described abolition of streptomycin activity against *Escherichia coli* and *Mycobacterium tuberculosis* by urea, uric acid, uracil, parabanic acid and various purine compounds. These substances were tested against dihydrostreptomycin, using *Bacillus subtilis* and *Staphylococcus aureus* as test organisms, and found to be inactive.

Rhymer, Wallace, Byers & Carter (1947) reported that soya-bean lipositol antagonized streptomycin, but I have been unable to confirm this with preparations made by these workers.

Sureau, Arquie, Boyer & Saviard (1948) described a 'streptomycinase' which was produced by *Pseudomonas aeruginosa* and by streptococci of Group D; Simkovics & Karossy (1948) also reported a 'streptomycinase' produced by *P. aeruginosa* and by *Escherichia coli*, *Salmonella typhi* and *S. paratyphi*. Linz (1948) described the antagonism of streptomycin by heat-treated suspensions of *Pseudomonas aeruginosa*.

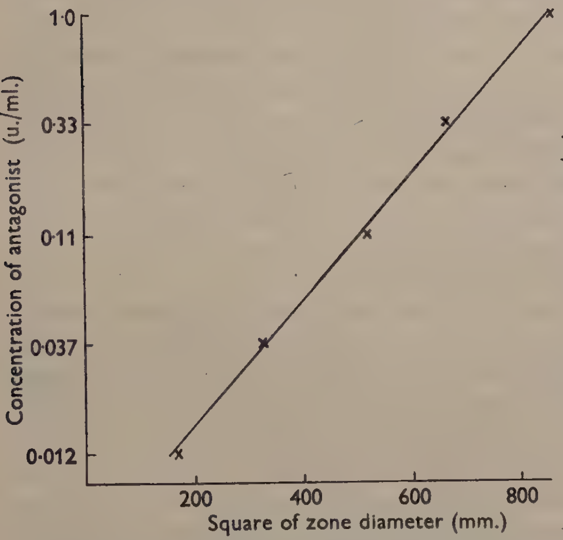
Lightbown (1950) reported that culture filtrates of *Pseudomonas aeruginosa* antagonized dihydrostreptomycin as well as streptomycin, and showed that the active factor was not an enzyme as implied by the term 'streptomycinase'. The active factor was shown to withstand boiling 0·1 N-HCl or NaOH, to diffuse through cellophan and to be extractable by lipid solvents. These properties were later confirmed by von Grumbach (1951). This paper describes the preparation and isolation of the antagonist in a crystalline form. It is probable that this antagonist is responsible for the 'streptomycin inhibition' activity of preparations of *P. aeruginosa* described by Bergman, Grubb, Bergström & Rösch (1954) at the time this paper was submitted for publication. It is interesting to note that these workers report that their preparations also antagonize the activity of neomycin.

Assay of antagonist

Standard preparation. Early in this work it was apparent that the activity of preparations of antagonist was markedly affected by such factors as pH value, temperature of incubation, nature of culture medium used in assay, and choice of test organism. In order to correlate measurements of activity obtained on different samples at different times, it was necessary to have a standard of antagonist activity. In the early experiments, a culture filtrate of *Pseudomonas aeruginosa* was set aside and to it was assigned an arbitrary potency of 1 u./ml. This unit of activity was used throughout the experiments; before the first standard sample was exhausted it was replaced by a second standard, which was assayed against the first. During 3 years the standard of activity has been passed through twenty consecutive standard preparations. The later ones have been prepared from the purest samples of antagonist available at the time, and the unit has been the activity contained in approximately 0·008 mg. of recrystallized material.

In the early experiments, samples were assayed by adding them to known solutions of dihydrostreptomycin in phosphate buffer, and measuring the residual antibiotic activity, by a modified cup-plate technique using fish spine beads (Humphrey & Lightbown, 1952). By this method a one-plate assay with sixty-four cups would just indicate a difference in activity of 30 % between two antagonist preparations containing approximately 1 u./ml. The activity of a preparation containing less than 0·3 u./ml. could not be measured.

An alternative and much more sensitive assay was developed in which cups containing the preparations of antagonist to be assayed were placed on plates of deep-seeded nutrient agar containing a concentration of dihydrostreptomycin sufficient to prevent growth of the test organism in the absence of antagonist. After incubation, sharply defined zones of growth appeared around the cups, and the potency of the samples assayed could be calculated from the diameters of the zones as in a normal antibiotic cup-plate assay.



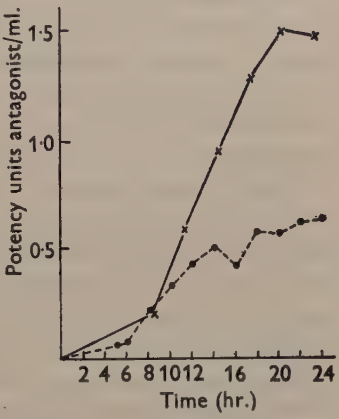


Fig. 1. Dose response curve for assay of antagonist by production of exhibition zones in nutrient agar containing dihydrostreptomycin and seeded with *Bacillus pumilus*.

Fig. 2. Effect of foaming on production of antagonist in stirred culture of *Pseudomonas aeruginosa*. Aeration constant (Wise, 1951) $1{\cdot}45^{-1}$. × —— ×, foaming controlled by 1·2 g. of silicone antifoam/l. of culture; ● - - - ●, foaming not controlled by 0·6 g. of silicone antifoam/l. of culture. Each curve represents the mean of values obtained from 6 replicate cultures.

Fig. 1 shows a typical dose response curve in which the square of the exhibition zone diameter is proportional to the log. of the concentration of antagonist. Since it was shown by Humphrey & Lightbown (1952) that the difference between linear and square relationship in cup-plate diffusion assays only becomes significant with very accurate assays, or when the dose levels are widely spaced, a linear relationship has been used in most of the assays. With a 6 × 6 Latin square design, i.e. 36 cups/plate, it is possible to detect a difference of potency of 6–10 % between two different preparations. For a given concentration of antagonist, the zone diameter is inversely proportional to the concentration of dihydrostreptomycin in the agar, but as the zone diameter increases the edge of the zone becomes less sharp. With the assay system normally used, giving reasonably sharp edges, it is possible to estimate activities down to 0·01 u. antagonist/ml.

The medium used in the assays was Wright infusion agar (Mackie & McCartney, 1946) containing 1 % of sodium dihydrogen phosphate and adjusted to a final pH value of 7·2. The test organism used was either *Bacillus subtilis* I.C.I. strain (NCTC 8236) or *B. pumilus* Mill Hill strain (NCTC 8241), seeded at a density of approximately 6×10^7 viable spores/ml. medium. Immediately before pouring the seeded agar, at a temperature of not more than 80°, dihydrostreptomycin sulphate solution was added to give a final concentration of from 8 to 24 μg./ml. The optimum concentration for each batch of medium had to be determined experimentally. Fish spine beads containing the assay samples were placed on the agar, after drying the plates at room temperature for 30 min., and the plates immediately incubated at 37° overnight. The temperature of incubation had to be carefully controlled, as a variation of $\pm 2°$ gave plates with either no growth at all or confluent zones. Much sharper zone edges were obtained when the assay plates were allowed to stand overnight at room temperature for diffusion of antagonist and subsequently incubated at 37°. Pl. 1, fig. 1, shows an assay plate which was obtained in this way. Zone diameters were measured on a screen after × 10 magnification.

Where possible, samples for assay were dissolved in phosphate buffer of pH 9·0 and $\mu = 0{\cdot}1$. Below pH 8·0, the solubility of the antagonist is approximately 10 μg./ml., whilst at pH 9·0 it is 20–30 μg./ml. Although the size of the zone diameter is affected by the pH value of the medium, it was found that the pH value of the test solution was not critical when the medium was buffered.

Samples dissolved in organic solvents were sometimes assayed by depositing a known small volume, e.g. 1 μl., on filter-paper and placing the filter-paper on the surface of the assay plate. The value of this method was limited by the low solubility of the antagonist and by the presence of inert lipid material which affected the transfer of the antagonist from paper to agar. Both these factors tended to cause an underestimation of activity, but in spite of this the method proved very useful.

Production of antagonist

Choice of strain. Thirty strains of *Pseudomonas aeruginosa* were compared, by a technique already described (Lightbown, 1950), for their power of producing antagonist. The strains were grown on the surface of cellophan sheet laid on nutrient agar containing dihydrostreptomycin and seeded with *Bacillus subtilis*. Large assay plates were used, which accommodated nine strains per plate. It was found more satisfactory to grow the strains within rings of soft paraffin deposited on the cellophan by means of a hot cork borer. In this way the area of growth of *Pseudomonas aeruginosa* was maintained constant and the zones of exhibition could be compared.

Twenty-three of the strains tested produced antagonist. Amongst these were strains which produced blue, yellow, green and red cultures. Some strains produced relatively large amounts of antibiotic material, which was made evident by a clear zone of inhibition inside the zone of exhibition. The strain finally chosen was a variant derived from the L III strain described by Don & van den Ende (1950). This organism produced a high yield of antagonist and relatively little antibiotic. It was maintained by daily subculture on nutrient agar slopes and dried by the method of Stamp (1947). The strain was very variable, and unless subcultured daily it was liable to lose completely or partially its power of production of antagonist. Examination of individual colonies showed wide variation in antagonist production from colony to colony; some colonies produced none at all. There was no evident correlation between cultural characteristics and antagonist production. Colonies which produced no antagonist sometimes gave rise to a small number of active colonies on repeated plating.

Culture medium. Good yields of antagonist were obtained on most of the complex culture media tested, for example, Wright infusion broth, Hartley digest broth, 1 % peptone water and C.C.Y. (Mackie & McCartney, 1946). The addition of a fermentable carbohydrate, which resulted in a culture with an acid pH value, decreased the yield; the highest yields of antagonist were found in cultures of pH 8–9 after overnight growth.

The chemically defined media described by Turfitt (1936) gave poor yields of antagonist. With ammonium acetate as the sole source of carbon and nitrogen, together with K_2HPO_4 and $MgSO_4$, quite good yields of antagonist were obtained, but the strain was very unstable, losing the power to produce antagonist when subcultured daily. The medium finally chosen was: ammonium lactate, 0·5 %; K_2HPO_4, 0·1 %; $MgSO_4.7H_2O$, 0·05 %; distilled water, adjusted to pH 7·5. The original L III strain grew well in this medium after two or three subcultures. By repeatedly plating on ammonium lactate medium, solidified with 1·0 % washed New Zealand agar, and testing isolated colonies by the cellophan technique and by assay of culture filtrates, a comparatively stable strain was obtained which gave higher yields in the ammonium lactate medium than in the more complex media. This strain was maintained in the dried state and could be revived at will with its properties unchanged.

Temperature of incubation was not critical between 30° and 37·5°, and a temperature of 35° was usually used.

The degree of aeration was found to be very important. Static liquid cultures gave very low yields or no activity, and the culture usually became very viscid. Aeration by shaking in conical flasks at 100 cycles/min., using the machine described by Kantorowicz (1951), gave consistently high yields. With this machine it is possible to vary the degree of aeration by using shelves at different levels, so altering the throw of the flask, or by altering the ratio of volume of culture to volume of flask. Highest yields (1–1·5 u./ml.) were obtained with the maximum throw of 4 in.; decrease of the throw to 2 in.

decreased the yield by 50 %. The optimum ratio of culture volume to flask volume was 1:8; an increase in this ratio to 1:5·7 decreased the yield by 40 %. Decrease of this ratio below 1:10 also decreased the yield, but it was not clear whether this was an aeration effect, since below this ratio evaporation losses with consequent concentration of the medium become troublesome.

When attempts were made to grow large single batches of culture, using other methods of aeration, very low yields were obtained. Growth in the apparatus described by Mitchell (1949) gave no antagonist at all. Growth in 20 and 10 l. aspirators aerated by Doulton filter candles (A.P.D. 7–10 μ) inserted through the tubulure gave irregular results. Yields of 0·4 u. antagonist/ml. were obtained, but the method was abandoned, mainly because of the difficulty of controlling foaming at high aeration rates, e.g. 2 vol. air/vol. medium/min. Lesser degrees of aeration gave consistently low yields.

The culture apparatus described by Fraser (1951) gave yields up to 0·7 u. antagonist/ml. with an aeration rate of 40 vol. air/vol. medium/min., but evaporation of the culture was a serious problem and the volume that could be handled was small.

With the aid of the Medical Research Council's Antibiotics Research Station at Clevedon, good yields were obtained with batches of 300 l. ammonium lactate medium. Preliminary experiments carried out in the 5 l. stirred culture vessels described by Kelly, Miller & Hale (1952) showed that there was an optimum aeration rate corresponding to an aeration constant (Wise, 1951) of 1·45 min.$^{-1}$. Under these conditions, potencies of 1–2 u. antagonist/ml. were regularly obtained. In cultures with aeration constants of 1·0 and 1·70, the potencies were lower (Table 1).

Table 1. *The maximum potency of antagonist (u./ml.) obtained in stirred flask cultures at different aeration constants (Wise,* 1951) *in the presence and absence of silicone antifoam.*

Each value represents the mean of several experiments, the number of experiments being shown in brackets.

	Aeration constant						
	2·6	1·7	1·45	1·0	0·84	0·7	0·25
	Potency (u./ml.) and number of experiments ( )						
Antifoam present	0·33	0·52	1·15	0·50	0·63	0·27	0·044
	(2)	(4)	(10)	(4)	(6)	(2)	(2)
Antifoam absent	0·21	—	—	—	0·11	0·31	0·039
	(2)				(2)	(2)	(2)

Foaming was controlled by using high concentrations (e.g. 1·2 g./l.) of D.C. Antifoam A (Dow Corning product); smaller concentrations were ineffective. The need to control foaming was not only the practical one of protecting the outlet filters, for it was found that low titres were always obtained in the presence of foam (Fig. 2). The effect on yield of antagonist of excess aeration and of foaming are partially explained by the fact that the antagonist is easily oxidized. Triamyl citrate also controlled foaming, but its use was abandoned because it increased the difficulty of extracting the active factor.

Length of incubation. The production of antagonist followed the growth curve of the organism; production ceased when the stationary growth phase was reached. At the higher aeration rates, continued aeration during the stationary phase of growth caused a decrease in potency.

The conditions for a typical 300 l. culture are given in the Appendix to this paper.

Methods of extraction and purification. Extraction by diethyl ether of culture acidified with HCl to pH 5·0 removed the antagonist but loss of activity was liable to occur during subsequent concentration by evaporation of the ether. The active factor was adsorbed from whole culture by charcoal, activated alumina (British Drug Houses Ltd.), calcium carbonate, kaolin and fullers earth, and could be eluted from all except carbon by 95 % ethanol.

The most satisfactory procedure proved to be extraction by *n*-butanol of culture acidified with HCl to pH 5·0. The volume ratio of *n*-butanol:culture was decreased to a minimum, i.e. 1:20 in order to decrease the amount of inert material extracted. With this ratio over 95 % of the activity could be removed by a single extraction. The butanol extract was concentrated by distillation in an atmosphere of oxygen-free nitrogen. In the presence of even a small amount of air, up to 50 % of the activity could be lost in the final stages of distillation when the residue became very concentrated, although in the earlier stages no loss occurred. The residue was a dark brown viscous oil.

Attempts to fractionate the brown oil by methods used in previous examinations of the products of *Pseudomonas aeruginosa* by Hays *et al.* (1945), and by Bergström, Theorell & Davide (1946) gave no useful results. Chromatographic separation on alumina columns and on paper, with water saturated butanol, as well as reverse phase chromatography using benzene and borate buffer pH 9·0, indicated the presence of two factors, both active in antagonizing streptomycin and both active inhibitors of bacterial growth. No separation of antagonist from associated antibiotic activity was obtained. On paper strips the activity frequently disappeared altogether, probably owing to oxidation. With the reverse phase benzene and borate buffer column, which was the most promising system, the quantities that could be handled were limited by the low solubility of the antagonist.

Fractionation depending on varying solubility of different constituents of the crude extract in organic solvents was successful. All the antagonist of the crude oil could be extracted by a small volume of hot benzene, and subsequent addition of light petroleum (80–100°) precipitated a large proportion of inert material, including most of the pigment, at a solvent ratio of 1 part of benzene to 2 parts of light petroleum. On standing, some of the antagonist also precipitated, but it was possible to remove the inert material first by centrifuging. This stage of the fractionation had to be carried out rapidly as with some preparations the active material began to precipitate after several minutes. The addition of a further 2 vol. of light petroleum to the supernatant precipitated most of the remaining antagonist. By repeating the procedure of precipitation from benzene with light petroleum several times, the antagonist with a potency of 100–120 u./mg. was obtained in a crystalline form (Pl. 1, figs. 2*a*, *b*).

The success of this method depended upon the proportion of antagonist to inert material in the crude butanol extract, and on the nature of the impurities. Table 2 indicates the variation in activity of the crude extracts obtained, by the use of different methods of cultivation and extraction. Although cultivation in a complex medium gave high potencies, the crude extract as might be expected contained a smaller percentage of activity. When the concentration of antagonist in the crude extract was less than 10 u./mg., the benzene and light petroleum fractionation was unsuccessful. For this reason, it was essential to use a culture medium of simple composition, and to extract as little inert material as possible in the initial extraction by using a minimum volume of solvent.

The crystalline material obtained as described was recrystallized from hot benzene, and from hot 50 % aqueous ethanol to a constant activity of approximately 125 u./mg. It was pale cream in colour, crystallizing as very thin rectangular plates from benzene, and imperfectly as sheaves of lanceolate plates from ethanol. Attempts to obtain large perfect crystals by slow crystallization were unsuccessful, usually resulting in oxidation with darkening and loss of activity. The melting point was 147°. Examination in a density column by the method of Low & Richards (1952) showed the material to be homogeneous with a density of 1·172. Analysis showed the presence of only the elements C, H, N and O, and gave values of C 75·0, H 8·4, N 5·0, corresponding to an empirical formula ($C_{17-18}H_{23-24}NO_2$). The material was stable in the dry crystalline state but was readily oxidized in solution. Its diffusion constant in 2 % agar was 0·0178 $cm.^2$/hr., which would suggest a molecular weight of the order of 300–400.

An examination of the ultraviolet absorption spectrum showed the material to be different from the pyocyanea compounds of Hays *et al.* (1945) although suggesting some similarity. An investigation of the chemistry of the crystalline material has been carried out by J. W. Cornforth and A. T. James (1954) who have reported that the material isolated is a mixture of several closely related components. One component has been tentatively identified as 2-heptyl-4-hydroxy-quinoline-*N*-oxide; synthetic material of this formula has approximately 30 % of the activity of the natural material isolated above; the second component is thought to be 2-(2′-nonenyl)-4-hydroxy-quinoline-*N*-oxide.

Biological properties

Although the mode of action of the antagonist has not yet been fully elucidated, the following biological properties are of interest.

Activity against streptomycin and dihydrostreptomycin. The activity of the antagonist against these two antibiotics is markedly affected by the pH value of the test system; the activity increases with decrease in pH value. Since activity of streptomycin decreases with decrease in pH value, this effect on antagonist activity might be expected. However, the increase in antagonist activity for a given decrease in pH value is greater than would be expected from the change in streptomycin activity.

The ratio of antagonist to antibiotic necessary to inhibit the activity of the

Table 2. *Activity of crude antagonist extracts obtained by different methods of culture and extraction*

Culture medium	Volume of culture (l.)	Conditions of aeration	Potency of culture before extraction (μ./ml.)	Method of extraction	Weight of crude extract of culture (mg./l.)	Units of antagonist/mg. of crude extract (μ./mg.)	% extraction of antagonist
Lemco peptone broth	0·250	Shaken	0·90	Ether; pH 2·0	240	1·3	33
Wright infusion broth	5	Shaken; 250 ml./2 l. flask	0·94	Fullers earth adsorption and elution with ethanol	150	3·0	48
Asparagine + salts	15 (3 × 5)	Filter candle in aspirator ½–1 vol. air/vol. cult./min.	0·1	Ether; pH 2·0	60	1·2	100
Ammonium acetate + salts	5	Filter candle in aspirator 2 vol. air/vol. cult./min.	0·27	Fullers earth adsorption and elution with ethanol	24	13·6	100
Peptone water	10	Shaken; 250 ml./2 l. flask	1·07	Single extraction into 1/10 vol. of *n*-butanol, pH 7·0. Emulsion cracked by centrifuging	230	4·0	87
Peptone water	10	Shaken; 250 ml./2 l. flask	1·07	Single extraction into 1/20 vol. of *n*-butanol, pH 7·0. Emulsion cracked by adding light petroleum	198	4·5	84
Ammonium lactate + salts	9·8	Shaken; 250 ml./2 l. flask	1·13	Single extraction into 1/20 vol. of *n*-butanol, pH 7·0. Emulsion cracked by centrifuging	120	9·0	82
Ammonium lactate + salts	5·3	Shaken; 250 ml./2 l. flask	1·7	Single extraction into 1/20 vol. of *n*-butanol, pH 7·0. Emulsion cracked by adding light petroleum.	98	15·0	85

dihydrostreptomycin has been determined in two ways. The concentration of antagonist at the periphery of a zone of stimulation, at the time at which the zone is fixed, may be calculated by the formula of Humphrey & Lightbown (1952) from data obtained in diffusion experiments. The concentrations so calculated for *Bacillus pumilus* in Wright infusion agar, buffered at pH 7·2, are of the order of 0·01–0·02 μg./ml. In these experiments the concentration of dihydrostreptomycin sulphate in the plates was 16 μg./ml. In the absence of antagonist, however, growth of the test organism only occurred at concentrations of less than 6 μg. dihydrostreptomycin sulphate per ml. Thus, under these circumstances, the antagonist is antagonizing 500 to 1000 times its weight of dihydrostreptomycin sulphate.

In the liquid chemically defined medium described by Roberts (1934) and with *Bacillus pumilus* as test organism, the antagonist antagonized 60–80 times its weight of dihydrostreptomycin sulphate at pH 7·2. In experiments of this type the end-point is fixed by a relatively small number of more resistant cells, which might explain the lower ratio.

Antibiotic activity of antagonist. Crude preparations of antagonist always inhibited growth when used in high concentrations, and it was at first thought that this antibiotic activity was probably due to contaminating antibiotic substances. Although this growth inhibitory effect decreased with purification, the crystalline preparation of antagonist could still inhibit growth. In liquid chemically defined medium of pH 7·2, seeded with *Bacillus subtilis*, the minimum inhibitory concentration of antagonist was about 5 μg./ml. compared with *c.* 50 μg. dihydrostreptomycin sulphate/ml. In buffered Wright infusion agar at pH 7·2, also with *B. subtilis* as test organism, the equivalent values were 0·6 μg. antagonist/ml. and 6 μg. dihydrostreptomycin sulphate/ml. The antibiotic activity of antagonist increased with decrease in pH value of the test system.

Effect on antagonist activity of previous exposure of test organism to dihydrostreptomycin. When a plate of seeded nutrient agar containing an inhibitory concentration of dihydrostreptomycin was incubated at 37°, and cups of antagonist were added at intervals of time after beginning incubation, no zones of exhibition were obtained around cups added after 4 hr. incubation (Fig. 3). It appears that the antagonist can only protect cells against the activity of dihydrostreptomycin and cannot reverse its bactericidal effect.

Shape and nature of zones of inhibition and exhibition. It is interesting to compare the shape and nature of the zones of inhibition and exhibition obtained in assays of dihydrostreptomycin and antagonist (Fig. 4, *a* and *b*, shows diagrammatically a vertical section of each type of zone). Dihydrostreptomycin is less active in the depths of the agar, resulting in a cup-shape zone of inhibition; with the antagonist, the zone is dome-shaped. In the inhibition zone, growth only occurs as a thin layer on the surface of the agar and then down the edge of the zone; in the exhibition zone, on the other hand, growth occurs uniformly throughout the depths of the agar. This ability to support growth deep in the agar is also seen when a solution of antagonist is placed on the surface of seeded agar which contains no dihydrostreptomycin. When a section of the agar is examined after incubation, it can be seen that in absence of antagonist the growth is in the surface layer, whilst in presence of antagonist growth occurs much more deeply (Fig. 4*c*).

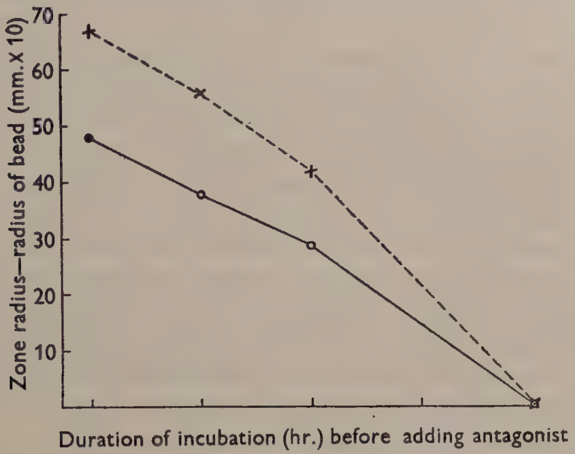


Fig. 3. Variation in size of exhibition zones produced by antagonist at 1 and 0·3 u./ml. added at intervals after beginning incubation of the assay plate at 37°. Test organism: *Bacillus pumilus*. Medium: buffered Wright infusion agar containing 16 μg. dihydrostreptomycin/ml. Each point represents the mean of 9 diameters; × - - - ×, 1 u. antagonist/ml.; O——O, 0·3 u. antagonist/ml.

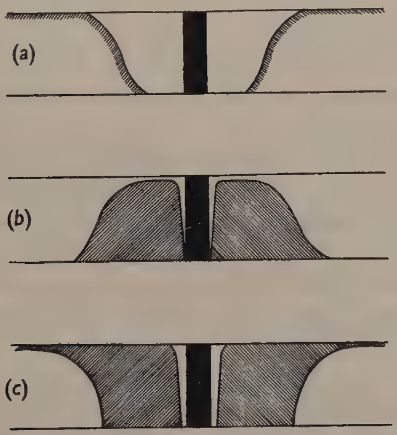


Fig. 4. Diagrammatical representation of the vertical section of zones obtained in punch hole plates, deep seeded with *Bacillus subtilis*. (*a*) With dihydrostreptomycin diffusing from the punch hole; (*b*) with antagonist diffusing into seeded agar containing dihydrostreptomycin; (*c*) with antagonist diffusing into seeded agar containing no dihydrostreptomycin. The shaded areas represent the distribution of growth in the agar and show how activity of dihydrostreptomycin and antagonist vary at different depths in the agar.

DISCUSSION

Apart from nucleic acid and some other complex anions, such as heparin, which are capable of precipitating dihydrostreptomycin and streptomycin from solution, the material described here is the only one known that will antagonize dihydrostreptomycin. Many substances which have been reported to have an effect against streptomycin have been tested by the methods used in this paper and have been found without activity against dihydrostreptomycin. Besides the compounds mentioned earlier, yeast extract, yeast nucleic acid, inositol, various components of the citric acid cycle, mercaptoacetic acid, cysteine, pyocyanin, α-oxyphenazine, a series of dicarboxylic acids (which it was thought might react with the two guanido groups of streptidine) and various substituted quinoline derivatives have been tested without success. The antagonist has no activity against penicillin or aureomycin.

The production of antagonist has been found only amongst *Pseudomonas* species. A number of other organisms has been examined, i.e. strains of *Staphylococcus aureus, Escherichia coli, Serratia marcescens, Bacterium bodenheimer, Bacillus subtilis* and *B. pumilus,* and several strains of streptococci of Lancefield's group D. With regard to group D streptococci, activity was reported from this organism by Sureau *et al.* (1948), but Rybak, Grumbach & Gros (1949) were unable to confirm this observation even with the original strain used by Sureau *et al.* (1948). Bearing in mind the ease of oxidation of the antagonist and the vigorous aeration necessary for its production by *Pseudomonas aeruginosa,* it may well be that the simultaneous production of reducing substances, e.g. pyocyanin, is necessary to allow accumulation of antagonist in the culture. It was noticed with *P. aeruginosa* that conditions of cultivation which produced a high yield of antagonist usually resulted in good pigment formation. The addition of dihydrostreptomycin to growing cultures of *P. aeruginosa* did not result in an increased yield of antagonist, nor did previous exposure of the *P. aeruginosa* to dihydrostreptomycin with resulting increase in resistance of the organism to this antibiotic.

The antagonism of dihydrostreptomycin activity has been studied only with *Bacillus subtilis*, *B. pumilus*, strains of *Staphylococcus aureus* and of *Escherichia coli*. With the first three organisms the antagonism is quite clear cut, but with *E. coli* the results obtained were equivocal. Of eleven different strains examined, ten (which were sensitive to dihydrostreptomycin) showed a very slight response to the antagonist in the presence of dihydrostreptomycin when tested in agar. The mechanism of the antagonism appears to be a biological one, and it is hoped that a further study of the properties of the antagonist may help to elucidate the mode of action of streptomycin. It seems unlikely that the antagonist will be of value in testing the sterility of samples of streptomycin and dihydrostreptomycin because of its low solubility and bacteriostatic activity in high concentrations, and because of its low activity against *E. coli*.

I wish to express my thanks to Dr J. H. Humphrey for his helpful encouragement and criticism during the course of this work; and to Messrs B. K. Kelly, G. A. Miller, C. W. Hale and Miss N. Smith of the Medical Research Council's Antibiotics Research Station, Clevedon, for their advice and assistance in carrying out the large-scale cultivation and extraction.

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EXPLANATION OF PLATE

PLATE 1

Fig. 1. Assay plate showing zones of exhibition produced by antagonist in nutrient agar deep-seeded with *Bacillus pumilus* and containing an inhibitory concentration of dihydrostreptomycin.

Fig. 2. Antagonist crystallized to constant activity from ethanol. (*a*) normal illumination; (*b*) showing birefringence in polarized light. × 300.

APPENDIX

LARGE-SCALE PREPARATION OF ANTAGONIST

Growth of 300 L. *Batch*

Inoculum

(1) An ampoule containing freeze-dried *Pseudomonas aeruginosa*, strain L III, was inoculated into 100 ml. 1 % Evans peptone (pH 7·0) in a 1 l. conical flask, and incubated on the shaker (84 oscillations/min., length of throw 7·5 cm.), at a temperature of 33–35°.

(2) 5 × 1 l. conical flasks, each containing 150 ml. ammonium lactate medium, were inoculated with 5 ml. of peptone culture and incubated on the shaker for 5 hr.

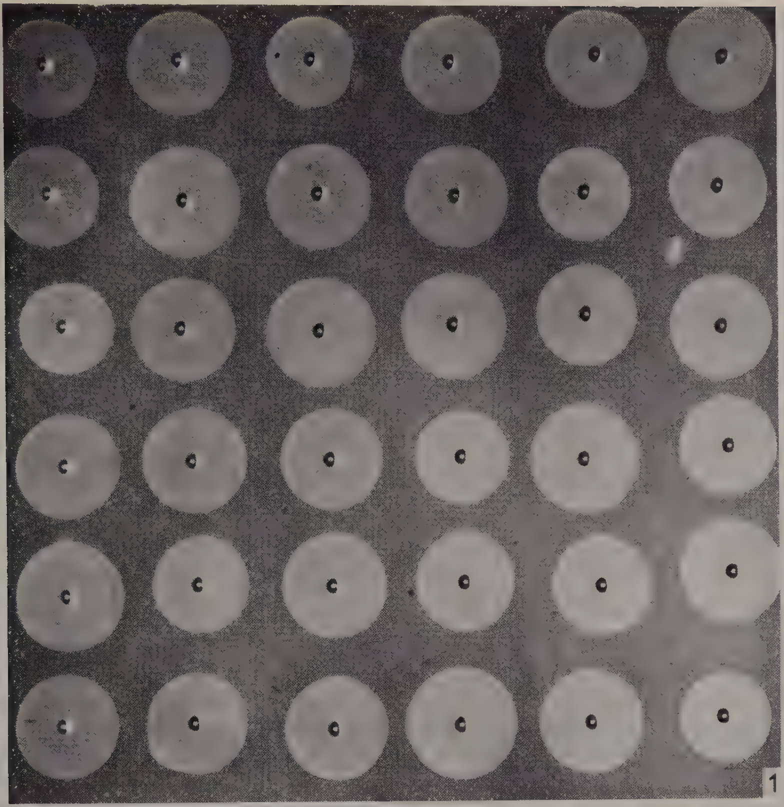
(3) The 5 hr. ammonium lactate cultures were bulked and 150 ml. volumes used to inoculate each of four stirred flasks each containing 4 l. ammonium lactate medium and 4·8 g. silicone D.C. antifoam (added before sterilization as 1/5 solution in chloroform). The stirred flasks were incubated at 33–35°, stirred at 600 r.p.m. and aerated with 9 l. air/min. for 5 hr. Conditions were equivalent to an ‘aeration constant’ (Wise, 1951) of 1·45 min.$^{-1}$.

(4) The 5 hr. stirred cultures were mixed together and used to seed the 100 gal. fermenter (Kelly *et al.* 1952).

Conditions for growth in 100 *gallon fermenter*

Medium	300 l. ammonium lactate medium (ammonium lactate, 0·5 %; K_2HPO_4, 0·1 %; $MgSO_4.7H_2O$, 0·05 %; distilled water to 300 l.; pH value adjusted to 7·2).	
Antifoam	200 g. silicone D.C. antifoam.	
Stirring	200 r.p.m.	Conditions to give ‘aeration constant’ = 1·45 min.$^{-1}$ (Wise, 1951).
Aeration	13–14 cu.ft./min.	
Temperature	33–35°.	
Duration of incubation	24 hr.	
Final pH value of culture	8·8.	
Potency of final culture	1·3 u. antagonist/ml.	

Foaming occurred after 9 hr. of incubation and was controlled by the addition of a further 50 g. silicone D.C. antifoam.



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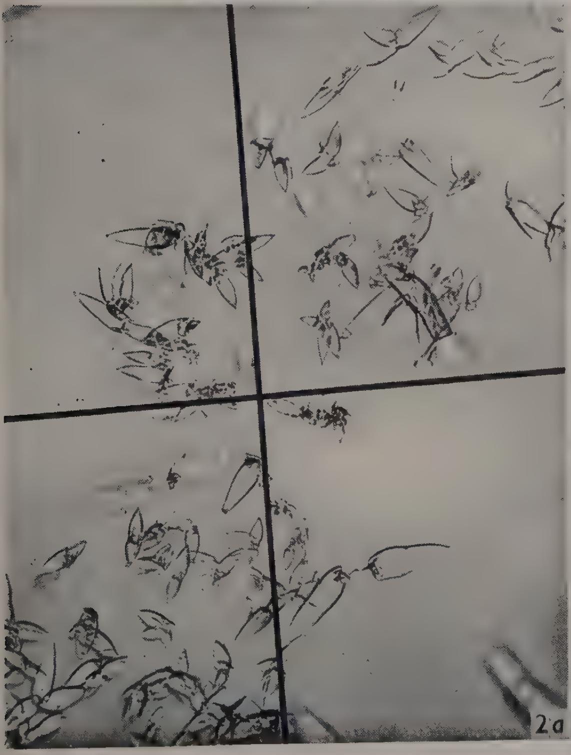

2a

2b

J. W. Lightbown. Streptomycin antagonist from *P. aeruginosa*

Extraction

Stage 1

Volume of culture	300 l.
Potency	1·4 u./ml.
Total units	420,000
pH value	8·9

Culture cooled to 24° and adjusted to pH 5·2 by addition of 500 ml. of conc. HCl (A.R.).

Added 45 l. of *n*-butanol and mixed by stirring at 200 r.p.m. for 1 hr. Intimate mixing was essential because of the low solvent : culture ratio; shorter time of contact did not give complete extraction.

Allowed to stand overnight.

Separated the two layers:—

Solvent emulsion.	Exhausted culture. Potency
Volume of emulsion	0·008 u./ml. Total units 2,400.
25 l.	Rejected

Added 10 l. of light petroleum (80–100°), mixed gently and allowed to stand 2 hr. (The addition of light petroleum cracks the emulsion, giving two clear liquid phases containing insoluble suspended matter.) The two layers were separated and the aqueous layer rejected. The solvent mixture layer was filtered through filter-paper pulp.

Volume 25 l. Total units 500,000.

The 25 l. was concentrated in an atmosphere of N_2 by using a van Heyningen vacuum still (1949); temperature $< 60^\circ$, to a volume of 1·5 l. Total units 450,000. If at this stage the butanol extract was saturated with water and stored at 4° overnight, silicone separated and could be rejected.

The remaining solvent was removed by distillation in a stream of oxygen-free nitrogen at atmospheric pressure, leaving a dark brown oil.

The oil (volume *c.* 50 ml.) was dissolved in 50 ml. warm benzene (A.R.), total units 312,000. (Some loss of activity, during the removal of last traces of solvent, appears unavoidable, but when nitrogen not freed from oxygen is used the losses are usually about 60 %.) This benzene solution was then treated as in the tabular description of Stage 2. (See p. 492.)

Stage 2

50 ml. benzene solution containing 100,000 u. Added 500 ml. light petroleum (80–100°). Allowed to stand overnight at 4°. Separated by centrifugation the precipitate formed

Precipitate

Oil, dark purple brown in colour. Weight 1–1·5 g. Total units 79,000. Activity 52–79 u./mg.

Supernatant

Volume 550 ml. Total units 5300

Dissolved in 50 ml. warm benzene. Added 100 ml. light petroleum (80–100°). Resulting precipitate immediately centrifuged and separated

Precipitate

Oil, dark purple brown in colour. Weight 720 mg. Total units 14,750

Supernatant

Added further 100 ml. light petroleum (80–100°). Immediate precipitation. Allowed to stand overnight at 4°. Filtered

Precipitate

Buff colour, crystalline. Washed with small vol. 1:4 benzene light petroleum mixture. Weight 658 mg. Total units 60,500. Activity 92 u./mg.

Supernatant

Volume 250 ml. Total units 5600

Dissolved in 50 ml. warm benzene. Added 100 ml. light petroleum (80–100°). Precipitate formed: immediately centrifuged and separated

Precipitate

Small amount oil. Dark purple brown. Not weighed or assayed.

Supernatant

Added further 100 ml. light petroleum (80–100°). Immediate precipitation. Allowed to stand overnight at 4°. Filtered

Precipitate

Pale yellow crystalline. Weight 464 mg. Total units 57,000. Activity 123 u./mg.

Supernatant

Volume 250 ml. Total units 6600

Recrystallization

From benzene: dissolved at 100 mg./ml. in hot benzene, filtered and allowed to crystallize. *Recovery:* 80–90 %.

From 33 % *ethanol in water:* dissolved in minimum volume of boiling aqueous ethanol (30 %), filtered and allowed to crystallize. *Recovery:* 80 %.

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A Comparison of the Properties of Penicillinase produced by *Bacillus subtilis* and *Bacillus cereus* with and without Addition of Penicillin

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SUMMARY: The properties (Michaelis constants, rates of inactivation at 100°, temperature coefficients and pH/activity relationships) of the extracellular penicillinase formed by a strain of *Bacillus subtilis* when growing in casein hydrolysate (= 'basal' enzyme) have been compared with those of the enzyme formed by the organism when growing in the same medium after addition of 1 unit benzylpenicillin/ml. (= 'induced' enzyme); no differences between the two preparations were detected. Similarly, no immunological or other differences were found between the basal and induced penicillinases of *B. cereus*, strain 569. However, the *B. subtilis* penicillinase was significantly different from the *B. cereus* penicillinase in the value of its Michaelis constant and the shape of its pH/activity curve; it was also quite distinct immunologically.

In most cases of microbial enzyme adaptation ('induced enzyme synthesis') the effect of the inducer is to cause a specific increase in activity of an enzyme which is, nevertheless, present to some extent in untreated cells. This spontaneously produced enzyme has been referred to as 'constitutive' or 'basal'; and, according to the enzyme and organisms concerned, may form a considerable or very minute fraction of that formed under influence of the inducer. In some instances no basal or constitutive enzyme activity has been detectable. With the β-galactosidase of *Escherichia coli* ML studied by Monod and his colleagues, Cohen-Bazire & Jolit (1953) showed that the trace of basal activity found in cultures grown in maltose could be explained by the known presence of a small proportion of mutant cells which were capable of forming large quantities of the enzyme constitutively. How far the property of producing an enzyme spontaneously (i.e. without added inducer) is distributed equally amongst a cell population, may in most cases be largely unknown, but in general, the process of enzyme adaptation must be regarded as an increase in the rate of production of an enzyme which is formed to some extent in the absence of the inducer, and not a formation *de novo*. The question is thus posed: Is constitutive (basal) enzyme composed of the same molecular species as induced enzyme, and if so, to what extent do the processes leading to their formation follow the same metabolic pathway?

In the work reported here an attempt has been made to obtain an answer to the first part of the question by a comparative study of the biochemical properties of penicillinases from a strain (749) of *Bacillus subtilis* and from a strain (569) of *B. cereus* formed (*a*) spontaneously, and (*b*) under the influence of benzylpenicillin. This problem has not been studied systematically before.

Reiner & Spiegelman (1947), however, claimed that the 'affinity constant' of galactozymase (O_2 uptake by intact cells of *Saccharomyces cerevisiae* in the presence of galactose) was over 8 times higher in unadapted cells than in those which had been previously adapted to galactose oxidation. It seems possible, however, that the O_2-uptake by unadapted cells in presence of galactose in these experiments, was not in fact due to galactose metabolism, but to the oxidation of traces of glucose and ethanol present in the sample of galactose used. It was shown by Lindegren & Palleroni (1952) that glucose and ethanol were present in samples of galactose prepared by the method used by Reiner & Spiegelman. Bernheim (1950), studying the adaptive sarcosine oxidase system in *Pseudomonas aeruginosa*, reported that the basal activity was unaffected by sodium dodecyl sulphate at concentrations which caused 32 % inhibition of enzyme in cells adapted by treatment with creatinine. He concluded that the adaptively-formed enzyme was on or near the cell-surface while the basal enzyme was inside the cell and so protected. Monod & Cohn (1952), however, found no differences, either immunological or biochemical, with respect to the relative activity on, or affinity for, different substrates, as between the constitutive (basal) and induced β-galactosidases of various strains of *Escherichia coli*. Indeed, in this case, there appeared to be no detectable immunological or biochemical differences between β-galactosidases produced by organisms in three separate bacterial genera (*E. coli*, *Shigella sonnei* and *Aerobacter aerogenes*), although the β-galactosidases formed by *Lactobacillus bulgaricus* and by *Saccharomyces fragilis* were distinct, both from the first group and from each other (see also Cohn & Torriani, 1953).

The penicillinases from the two species of *Bacillus* chosen for this work were especially suitable because (*a*) the enzymes are released into the culture fluid and can be conveniently studied in a cell-free state without further treatment; (*b*) although the inducer (penicillin) markedly and specifically stimulates enzyme production—causing a 10- to 20-fold increase with *B. subtilis*, strain 749, and a 200- to 500-fold increase with *B. cereus*, strain 569—there is an accurately measurable amount of basal activity (especially in organism 749) present in the control untreated culture; (*c*) it seems reasonable to assume that the basal activity is really produced by the cells spontaneously, since there can hardly be traces of penicillin in the casein hydrolysate or peptone medium used.

Ideally, a comparison of two similar enzymes should be made with fully purified preparations. This would not only allow a more accurate comparison, but extend the range of properties that could be compared. However, although the induced enzyme of *Bacillus cereus*, strain 569, has been purified (Pollock & Torriani, 1953), the immediate chances of obtaining enough of the basal enzyme of either species (where the amount produced is not more than 1/100 of that formed by organism 569 after induction) in a fully purified state are obviously remote. The next best alternative thus appeared to be the use of simple cell-free supernatant fluids from cultures grown in the same medium and, as far as possible, under the same conditions for both basal and induced enzyme preparations.

METHODS

Organisms

(*a*) ***Bacillus subtilis***, strain 749, kindly sent by Mr Leeson of the Distillers Co., Liverpool. This organism (under the label 'B') was used in previous studies (Manson & Pollock, 1953) on the thermostability of different penicillinases, but was erroneously classified as a strain of *B. cereus* (Leeson, 1950). Its small cell size and the fact that we found it would grow on NH_3 as sole source of N and without added growth factors indicate that it should be regarded as a *B. subtilis*.

(*b*) ***B. cereus***, strain NRRL 569, used previously for studies on penicillinase adaptation.

Media and materials

Penicillin: crystalline sodium benzylpenicillin from Glaxo Laboratories Ltd.

Gelatin: Difco 'standardized'.

Casein hydrolysate medium (CH): 0·8% (w/v) acid hydrolysed 'vitamin-free' casein (Allen and Hanburys) supplemented by M/600 $MgSO_4$, M/50 sodium-potassium phosphate buffer (pH 7·0), M/12,500 tryptophan and M/5000 cystine.

'S' broth: 1% (w/v) peptone (Evans) + 0·3% (w/v) Lab-Lemco (pH 7·0), prepared as described by Pollock & Perret (1953).

Tryptic digest broth (Hartley, 1922); CCY medium (Gladstone & Fildes, 1940).

Penicillinase assay

Except in the pH/activity experiments, the enzyme was assayed at 30° in bicarbonate/CO_2 buffer at pH 7·0 by the manometric method of Henry & Housewright (1947). No corrections were made for the slight degree of CO_2 retention which occurs, since the flask constants did not vary enough for such retention to affect comparative results. For pH/activity experiments the iodimetric method of Penau, Phillipe & Benoist (1951), as modified and improved by Perret (private communication), was used, since a wide range of pH values is not attainable by the manometric method. The iodimetric method estimates directly the penicilloic acid formed. When all the necessary controls are used in order to correct for spontaneous decomposition of penicillin, this method can be extremely accurate and is particularly useful for the detection of differences in the low enzymic activities found at pH values some way from the optimum. All activities are expressed as μmole penicillin destroyed/hr. at 30° (= penicillinase units as defined by Pollock & Torriani, 1953).

Penicillinase production

Bacillus subtilis, strain 749. (*a*) Basal enzyme. In order to obtain enough enzyme in the cell-free culture fluid, one-fifth of the cells from a spore-inoculated nutrient agar slope, incubated for 16 hr. at 35°, were used to inoculate 50 ml. of CH medium which was then shaken aerobically at 35° for 24 hr. The cells were immediately centrifuged off and the supernatant fluid, having an activity of from 5 to 10 penicillinase units/ml., was adjusted to pH 7·0 and stored at 2°. (*b*) Induced enzyme. Cells from a spore-inoculated liquid CCY medium shaken for 24 hr. at 35°, were washed and shaken in 50 ml. CH medium containing 1 unit penicillin/ml. at an initial opacity corresponding to 1·5 mg. dry-wt. bacteria/ml., at 35° for 3·5 hr. The penicillinase activity in the supernatant fluid, after removal of cells, was 15–30 units/ml. The preparation was treated and stored in the same way as the basal fraction. Both the total and exocellular penicillinase activities of these induced cultures were about 7 times those obtained in cultures similarly prepared but without addition of penicillin. It is thus reasonable to conclude that the maximum possible proportion of basal enzyme present in the 'induced' preparation was 15 %.

Bacillus cereus, strain 569. (*a*) Basal enzyme. 50 ml. of 'S' broth were inoculated with 1 drop of a spore suspension containing 3×10^8 viable cells/ml., and incubated at 35° on a shaker for *c*. 6 hr., by which time cell growth was equivalent to 1·0 mg. dry weight cells/ml. The cells were centrifuged off and the supernatant fluid kept at 2° after addition of gelatin to 1 % (final) to stabilize enzyme. The penicillinase activity of this fluid was usually about 1 unit/ml. and it was therefore concentrated, when higher activities were necessary, by freeze-drying. (*b*) Induced enzyme was prepared in exactly the same way, except that penicillin (1 unit/ml.) was added when the cell concentration was equivalent to 0·1 mg. dry wt. bacteria/ml. Activities reached values up to 500 units penicillinase /ml.

RESULTS

Inactivation by heat

Loss of enzyme activity at boiling-water bath temperature was followed by the technique of Manson & Pollock (1953). It had been found that gelatin or high molecular weight substances present in tryptic digest broth afforded considerable protection. Basal and induced enzyme preparations from *Bacillus subtilis*, strain 749 grown in nutrient broth, as well as preparations from cultures grown in CH medium, were examined (Table 1). No significant differences in the rates of inactivation of the basal and induced enzyme preparations were found, and the degree of protection afforded by the presence of nutrient broth was the same for both.

Previous work (Manson & Pollock, 1953) revealed some apparently signifi-

Table 1. *Rates of inactivation at* 100° *of induced and basal penicillinase of* Bacillus subtilis, *strain* 749

		% original activity remaining after treatment at 100° for			
Growth medium	Enzyme	2 min.	5 min.	16 min.	60 min.
Casein hydrolysate	Induced	31	19	11	7
	Basal	40	27	18	—
Broth	Induced	75	85	70	50
	Basal	85	83	72	42

cant differences in rate of thermal inactivation of penicillinase preparations from different organisms, and in the protective effect of added gelatin or broth. For instance, the penicillinase of *Bacillus subtilis*, strain 749, is only 20 % inactivated after 5 min. at 100° in the presence of 1 % gelatin or tryptic digest broth, while the enzyme of *B. cereus*, strain 569, is 55 % destroyed under similar conditions. It is, however, possible that the smaller protective effect of gelatin for penicillinase from *B. cereus*, strain 569, may be due, in part at least, to the known presence of a gelatinase (not detected in *B. subtilis*, strain 749). For this reason, no heat inactivation studies were done with *B. cereus*, strain 569.

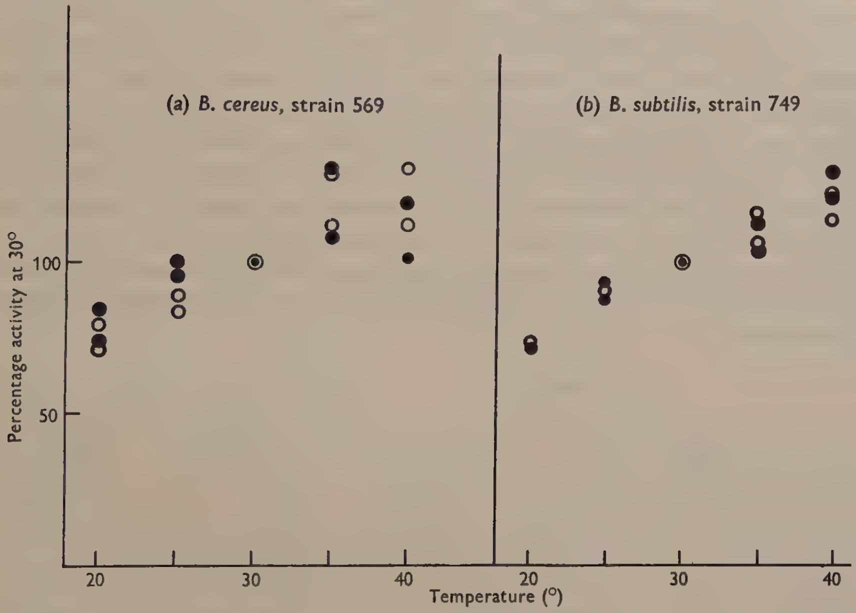

Fig. 1. Relationship between penicillinase activity and temperature for basal (●) and induced (○) enzyme preparations from (*a*) *B. cereus*, strain 569, and (*b*) *B. subtilis*, strain 749, expressed as percentage of respective activities at 30°. Each point is the mean of duplicate estimations made on each of two batches (plotted separately) of each enzyme, prepared on different occasions.

Temperature coefficient

Activities of the two preparations from both organisms at temperatures between 20° and 40° were compared after diluting the induced enzyme with fresh medium to give the same activity as the basal enzyme and adding gelatin to 1 % to prevent possible inactivation at the higher temperatures. The activity curves (Fig. 1), representing results with two preparations of basal and induced enzyme from both organisms, show no significant differences either between basal and induced enzymes, or between the enzymes from the two different species.

pH/*activity curves*

All preparations were diluted in 0·5 % gelatin to give an activity of *c.* 3·0 units/ml. (as measured at pH 7·0 by the manometric method) and their activities at 30° compared between pH 4·0 and 8·0 using M/5 citrate buffer for the pH range 4·0–6·0 and phosphate buffer for pH 6·0–8·0, so that overlap with the two different buffers was obtained at pH 6·0. Fig. 2*a, b* shows the curves obtained when activities are expressed as percentages of the maximum (at pH 6·0). Measurements at pH 4·0 with enzyme from *Bacillus subtilis*, strain 749, were inevitably rather inaccurate because of (*a*) the very low absolute activities, and (*b*) spontaneous decomposition of penicillin to penicilloic acid was 4 times that due to enzymic hydrolysis. In all other cases duplicates agreed to within 5 % of the mean. A repeat experiment, using different batches of all four enzyme preparations, gave curves which were indistinguishable from those of the first experiment. It was confirmed, by titrating an enzyme-inactivated sample of penicillin after incubation at different pH values, that decomposition of penicilloic acid (which reacts specifically with the iodine and is thus assayed directly in this method), even at pH 4·0 was not sufficient to interfere with results. It can be seen that while the curves for penicillinases from the two species are significantly different on the acid side of neutrality, there is very little difference between basal and induced enzyme in either case.

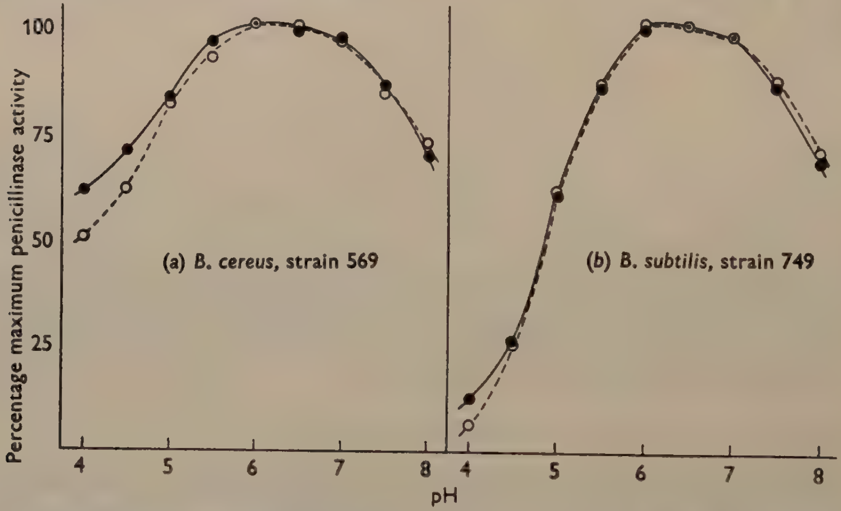

Fig. 2. Relationship between penicillinase activity and pH for basal (—●—●—) and induced (—○—○—) enzyme preparations from (*a*) *B. cereus*, strain 569, and (*b*) *B. subtilis*, strain 749, expressed as percentage of their respective maximal activities (at pH 6·0). Each point is the mean of duplicate estimations of activity.

Affinity constants

Preliminary tests soon indicated that the Michaelis constant of penicillinase was too low to permit even an approximate estimation by the direct manometric technique. Attempts to estimate low concentrations of penicillin by means of the cup assay were also unsuccessful since it was found necessary to inactivate the enzyme in order to obtain reproducible results, and this could not be done (e.g. by heat) without considerable loss of penicillin.

An alternative method based on the rate of penicillinase formation by *Bacillus cereus*, strain 569, in response to submaximal quantities of penicillin, was therefore evolved. It was found (Pollock, 1952) that there is a fairly good linear relationship between the rates of penicillinase formation by a log-phase culture of *B. cereus*, strain 569, growing in broth and the concentrations of penicillin added as inducer, within the range 0·01–0·10 units penicillin/ml. The 'primary interaction' between penicillin and cells which initiates this enzymic induction is so rapid (50 % complete in 60 sec.) that it is uninfluenced by the penicillin-destroying effect of small amounts of penicillinase added at the time and thus affords a means of estimating very low penicillin concentrations in the presence of penicillinase. There is thus no need to add any enzyme inactivator.

The enzyme sample was diluted in 'S' broth containing 1 % gelatin to such a degree that, when diluted 1/10, about half the penicillin at the particular concentration being tested would be destroyed in 45 min. at 30°. At intervals of 15 min., 1 ml. samples of the enzyme solution were added to four separate tubes containing 9 ml. of the appropriate concentration of penicillin in 1 % gelatin 'S' broth. All tubes were removed from the bath at the time of addition of enzyme to the fourth tube and diluted, when necessary, so that the final penicillin concentrations fell between 0·04 and 0·20 unit/ml. At the same time two known concentrations of penicillin in 1 % gelatin 'S' broth were prepared containing 0·04 and 0·20 unit/ml.

The test culture itself was prepared by inoculating 50 ml. 'S' broth with 1 drop of a standard spore inoculum (3×10^8 viable cells/ml.) of *Bacillus cereus*, strain 569, and shaking at 35° until the cell concentration was equivalent to *c.* 0·4 mg. dry wt./ml. This took 4–4·5 hr. Gelatin was added to a final concentration of 1 % and 5 ml. lots were placed in six 50 ml. conical flasks shaking in a 35° water-bath. At this point 5 ml. of unknown or standard samples, prepared as above, were added as rapidly as possible, each to one flask of test culture. Shaking of the flasks was continued and 2·5 ml. samples were transferred at 30 and 60 min. into iced oxine for assay as in an ordinary adaptation experiment (Pollock, 1952). Penicillinase activities were measured manometrically and the rates of enzyme formation (difference between 60 and 30 min. values) calculated. The penicillin concentrations are directly proportional to these and can thus be read off on the standard curve. The rates of penicillin destruction were measured as the mean rate over the period when not more than half the penicillin had disappeared.

In practice this very unorthodox method gave reasonably consistent and accurate assays of penicillin. Thus rates of penicillinase formation following induction at a penicillin concentration of 0·10 unit/ml. showed a variation of ±7·5 % about the mean in a series of five identical flasks. One major advantage, for calculation of the Michaelis constant, is that it is possible to measure rates of penicillin hydrolysis over a wide range of initial penicillin concentrations which are well below enzyme saturation. The constants have been calculated by direct substitution of the velocity found at the given initial penicillin concentration, in the Michaelis formula:

$$V = \frac{V_{\text{max.}} \times [S]}{[S] + K_m},$$

where V is the reaction velocity at substrate concentration S, $V_{\text{max.}}$ is the velocity when the enzyme is saturated by substrate, and K_m the Michaelis constant. $V_{\text{max.}}$ was measured, by the same technique, using an initial penicillin concentration of 500 units/ml. ($8{\cdot}5 \times 10^{-4}$ M) and an enzyme activity of 0·5 unit penicillinase/ml., giving results within 10 % of those obtained by the manometric technique, with a penicillin concentration of 3300 units/ml.

The constants, thus calculated, using initial penicillin concentrations of 0·2–200 units/ml., vary at most 59 % above to 29 % below the mean, including the least accurate value (obtained at 200 units/ml.), when the enzyme of *Bacillus subtilis*, strain 749, is 60 % saturated. Table 2 shows the results obtained. As with the pH/activity curves the similarity of the values for basal and induced enzyme preparations from the same strain is made more striking by the significant difference ($P = < 0{\cdot}001$) between the enzymes of the two species.

Table 2. *Michaelis affinity constants of induced and basal penicillinases of* Bacillus subtilis, *strain* 749, *and* Bacillus cereus, *strain* 569

Organism	Enzyme preparation	Penicillin concentration (units/ml.)		Michaelis constant (units/ml.)
B. subtilis, strain 749	Induced	200		159
		20		82
		2		133
		0·2		91
			Mean	116 ($=1{\cdot}98 \times 10^{-4}$ M)
	Basal	200		165
		20		79
		2		94
		0·2		77
			Mean	103 ($=1{\cdot}76 \times 10^{-4}$ M)
B. cereus, strain 569	Induced	20		46
		2		32
		0·2		33
			Mean	36 ($=6{\cdot}2 \times 10^{-5}$ M)
	Basal	20		30
		2		30
		0·2		55
			Mean	38 ($=6{\cdot}5 \times 10^{-5}$ M)

The Michaelis constants were calculated by direct substitution of measured velocities for each substrate concentration (for details, see text).

Immunological properties

After most of this work was complete, a specific neutralizing antiserum to induced penicillinase of *Bacillus cereus*, strain 569, was obtained by repeated injections of large amounts of purified enzyme into rabbits. Full details of the technique will be published later. This anti-penicillinase serum caused *c.* 95 % inhibition of the penicillinase of *B. cereus*, strain 569, and gave constant ratios of antiserum added: penicillinase neutralized, when enzyme was in excess, at all concentrations of enzyme or antibody, irrespective of the medium. By contrast, a sample of the induced penicillinase of *B. subtilis*, strain 749, was completely uninhibited by 5 times the dose of antiserum necessary to neutralize the equivalent amount of enzyme from *B. cereus*, strain 569. The penicillinases from the two species are therefore immunologically distinct. However, the induced enzyme from *B. cereus*, strain 569, was inhibited to the same extent by a given amount of antiserum as was the basal enzyme from the same organism (see Fig. 3).

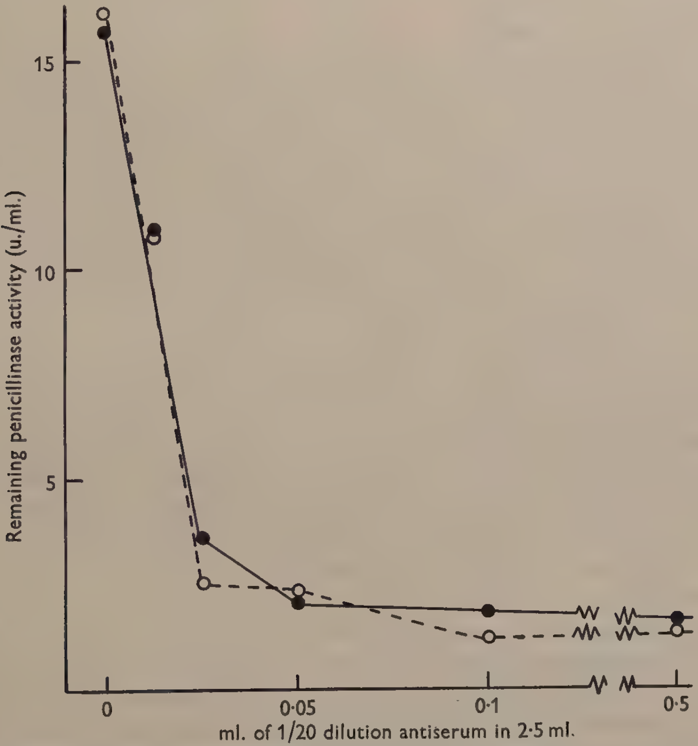

Fig. 3. Neutralizing effect of rabbit antiserum prepared against purified *B. cereus*, strain 569, induced penicillinase, when mixed with samples of *B. cereus*, strain 569, basal (—●—●—) and induced (—○—○—) penicillinase.

Ratio of activities on n-*heptylpenicillin and benzylpenicillin*

Henry & Housewright (1947) reported that the ratio of the rate of hydrolysis of *n*-heptylpenicillin (Penicillin K) to that of benzylpenicillin (Penicillin G) by *Bacillus cereus*, strain 569, was from 0·59 to 0·69. This ratio was therefore measured for the basal and induced penicillinases of both *B. subtilis*, strain 749, and *B. cereus*, strain 569, prepared as described above. The results, summarized in Table 3, indicate no difference in the ratio obtained with any of the four preparations. The n-heptylpenicillin was kindly supplied by Dr J. H. Humphrey and contained only 10 % of other penicillins (mainly penicillin dihydro F).

Table 3. *Relative activities of induced and basal penicillinases of* Bacillus subtilis, *strain* 749, *and* Bacillus cereus, *strain* 569, *on penicillin K and G*

		Ratios of $\frac{\text{activity on pen. K}}{\text{activity on pen. G}}$ at substrate concentration		
			$2{\cdot}8\times10^{-3}$M	
Organism	Enzyme	$1{\cdot}4\times10^{-3}$M	Exp. I	Exp. II
B. subtilis, strain 749	Basal	0·55	0·54	—
	Induced	0·61	0·61	—
B. cereus, strain 569	Basal	0·58	0·64	0·65
	Induced	0·60	0·64	0·69

Test for presence of constitutive mutants in inducible cultures

The question posed in the introduction changes its nature somewhat if the amount of basal activity is only a reflexion of a greater or smaller proportion of genetically distinct constitutive mutant cells present in the inducible population under study. It would then be a matter of whether the penicillinases of two genetically distinct but very closely allied strains were of different molecular species and thus possibly produced by different mechanisms: and this might be considered less surprising than the possibility of two separate mechanisms controlling the formation of penicillinase in the same cell. Although such an explanation for basal activity has been proved reasonable for the β-galactosidase of *Escherichia coli*, ML strain, where the basal/inducible activity ratio is only 0·10 % (Cohen-Bazire & Jolit, 1953), it is rather less likely for the penicillinase of *Bacillus cereus*, strain 569, where the minimum ratio found is 0·5 % and much more improbable for nitratase of *E. coli*, strain 1433 (ratio varying from 3 % in washed suspensions without N source to 30 % in high concentrations of casein hydrolysate—Wainwright, 1950), or for penicillinase of *B. subtilis*, strain 749, with a ratio of 5 to 10 %. Even allowing for the possibility that constitutive mutant strains might produce, say, 10 times more enzyme than maximally induced adaptive strains, it would still mean an incidence of 1 constitutive mutant in a total of 2000 cells for cultures of *B. cereus*, strain 569, and 1 in 100 for *B. subtilis*, strain 749. Such a high proportion of mutants, if present, should be detectable without much difficulty. It might be considered that the best method for demonstrating the existence of constitutive mutants would be one analogous to that used by Cohen-Bazire & Jolit (1953)—viz. an attempted selection by alternate subcultures in media respectively with and without inhibiting concentrations of penicillin. However, an attempt to do this with *B. cereus*, strain 569, by 50 passages in broth, containing 100 units/ml. of penicillin on every alternate subculture, gave no significant increase in basal enzyme. The original intention was to give any cells producing penicillinase in large quantities without penicillin a slight selective advantage over the inducible cells when the culture was transferred to penicillin. It can, however, be argued that the production of penicillinase, particularly if exocellular, is a collective bacterial weapon against penicillin and might confer little or no selective growth advantage to the cells that form it, since its protective effect would be largely, if not entirely, shared by all cells in the culture. The following test was therefore devised in order to look for possible constitutive mutants.

Sandwich layer plates of CCY medium containing 1 vol. in 25 of a 0·5 % aqueous solution of acid fuchsin, decolorized with NaOH (Andrade's indicator), were poured as follows: lower layer, 15 ml. containing 1·5 % agar; middle layer, 7 ml. containing 3 % agar + 1 ml. bacterial spores under test; upper layer, 7 ml. containing 3 % agar. Plates were incubated overnight at 30° and the following morning 300,000 units of penicillin in 2 ml. of water were added to the surface of each plate. Positive colonies producing large quantities of penicillinase constitutively, turn a bright red after about 15 min. at room temperature. Negative colonies (i.e. non-penicillinase-producers or producers of penicillinase in large quantities only after induction by penicillin) remain colourless for several hours. 'Inducible' colonies can easily be distinguished from non-producers by incubating with a small quantity (100 units/plate) of penicillin at 35° for 4 hr., after which the further addition of the full 300,000 units results in their going red within 15 min., like 'constitutives'. The efficacy of this test was checked with a constitutive-penicillinase mutant isolated by Dr P. H. A. Sneath from a strain of *Bacillus cereus* closely related to *B. cereus*, strain 569. This mutant (*B. cereus* 5/B) forms about twice as much penicillinase constitutively as does *B. cereus*, strain 569, under conditions for maximal adaptation. Artificial mixtures showed that it was easy to detect single colonies of *B. cereus* 5/B, by the above technique, against a background of up to 1000 colonies per plate of either *B. cereus*, strain 569, or *B. subtilis*, strain 749.

By this means, therefore, it was possible to examine 25,000 colonies of *Bacillus subtilis*, strain 749, and 16,000 colonies of *B. cereus*, strain 569; but not a single 'positive' colony of a constitutive penicillinase-producer was found.

DISCUSSION

It appears *a priori* rather unlikely that a homogeneous population of cells would produce the same highly complex protein, having a specific and presumably very restricted metabolic function, by two completely divergent pathways. The probability that the mechanism for its production is basically the same under two different conditions thus depends to a large extent on the demonstration that its properties, whatever the conditions of its formation, are constant.

The impossibility of obtaining large amounts of purified basal enzyme has meant that no comparison of properties such as amino-acid composition, electrophoretic mobility, molecular weight, turnover number or solubility in salts, etc., has been feasible; and it can always be argued that two apparently similar substances do in fact differ by some property which it has been impossible to study. Nevertheless, most catalytically similar enzyme preparations from even closely related types of cells show at least some immunological differences (see Sevag, 1945; Marrack, 1950); and, in the present work, it was not difficult to demonstrate fairly marked differences in pH/activity curves, Michaelis constants and immunological reactions between the penicillinases of two distinct species of the same genus. Even in the case of triose phosphate dehydrogenase studied by Velick & Udenfriend (1953) where it was shown that, although the purified enzyme obtained from rabbit muscle had the same molecular weight and amino-acid composition as that extracted from yeast, the two were quite distinct immunologically and had different affinity constants for combination with diphosphopyridine nucleotide.

Thus, where the mechanism of formation of similar enzymes differs from one case to another, there seems to be a reasonable chance, even within the limited range of tests used here, of detecting differences in the properties of the product.

In this work no significant differences have been found between the basal and induced penicillinase fractions produced by either *Bacillus subtilis*, strain 749, or *B. cereus*, strain 569. This supports the hypothesis that the mechanisms for the synthesis of basal and induced enzyme are fundamentally similar. In its simplest form such a hypothesis implies that the only difference lies in the extent to which the cells can themselves synthesize the necessary specific factor which evokes enzyme production; and that after the initial stages of inducer assimilation and metabolism the paths of synthesis are identical.

A special case of this hypothesis, put forward by Cohn & Monod (1953) and by Pollock (1953), is that basal (constitutive) enzyme production is under the catalytic control of an endogenously produced 'organizer' whose synthesis in certain cases may be specifically augmented by addition of the exogenous inducer. It predicts that no consistent difference will be found between basal and induced enzyme in the same organism. In the case of penicillinase production by *Bacillus cereus* and by *B. subtilis* this prediction has so far been found to be true. The more evidence accrues, from the study of other systems and organisms, to confirm the similarity between basal and induced enzyme, the more important does it become to continue the search for a common pathway in their mode of production.

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The Fluorescent Materials produced *in vivo* by certain Dermatophytes

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SUMMARY: The fluorescent materials present in human and cat hair as a result of infection with *Microsporon canis*, *M. audouini* and *Trichophyton schoenleini* were extracted with dilute ammonia. Paper chromatography and electrophoresis showed that the principal fluorescent material was common to all these infections and that further fluorescent substances were present in the extract from *T. schoenleini* infected hair. Chemical properties and absorption spectra of the extracts have been examined.

The brilliant green fluorescence in Wood's light shown by hairs infected with *Microsporon canis*, *M. audouini* and *Trichophyton schoenleini* was first noticed by Margarot & Devèze (1925) and is now an accepted method of diagnosis in cases of small-spore ringworm, although the nature of the fluorescent material has not been determined. Davidson & Gregory (1932) showed that it could be readily extracted from the hairs by hot water and, working with a crude extract, they showed the presence of a substance or substances containing nitrogen, phenolic and aldehyde groups but no ninhydrin-reacting substances. Felsher (1949) extracted the hairs with cold 2N-sodium bromide and showed colour changes of the fluorescence with pH, and quenching by a number of salt solutions. Robinson, Figge & Bereston (1953) described spectroscopic studies made on an aqueous extract of hair infected with *Microsporon audouini*; they purified their extract by shaking with a mixture of alumina, aluminium silicate and talc under unspecified conditions. Infra-red spectra showed the presence of material containing a substituted amide group, hydroxyl, methyl and amino groups. The present work describes a comparison of the fluorescent substances produced by the three fungi, and indicates certain common products and the presence of additional fluorescent materials in the extract from the *Trichophyton schoenleini* infection. Isolation of the pure substances has not been achieved, principally owing to the very small amounts available.

MATERIALS AND METHODS

Infected hair. This was obtained from patients in Leeds and Huddersfield and also in the case of infections due to *Microsporon canis* from the hair of cats infected with *Microsporon*. Hair was also obtained from a case of favus which occurred in an Italian immigrant. Mycological identification of the fungus was made in all cases.

Extraction of the fluorescent material. Infected hair was extracted with ether for 24 hr. in a Soxhlet extractor to remove fatty materials. Boiling with $1{\cdot}17\,\text{N-NH}_3$ for 20 min. gave complete extraction of the fluorescent substance. This method was preferred to extraction for 1 hr. with boiling water as employed in the other studies cited, since this was not always found to give complete extraction. The ammoniacal extract was immediately concentrated to dryness *in vacuo* at 40°. The material was maintained in the dry state at −20° since it was found that fluorescence faded steadily on keeping in aqueous solution.

Chromatography. The method of Consden, Gordon & Martin (1944) was used with Whatman papers nos. 3 and 4 previously washed with water and equilibrated in the presence of the solvent mixture before development of the chromatogram. The solvent mixtures which proved of the greatest value in separation of the fluorescent materials were *n*-butanol (50 vol.) + glacial acetic acid (10 vol.) + water (40 vol.) and *n*-butanol (40 vol.) + ethanol (40 vol.) + ammonia solution sp.gr. 0·880 (4 vol.) + water (16 vol.). The butanol was rendered non-fluorescent by shaking with norite and re-distilling, and butanol/acetic acid/water mixtures were utilized after 48 hr. equilibration at room temperature. When necessary chromatograms were sprayed with 0·1 % ninhydrin in purified *n*-butanol.

Paper electrophoresis. The method of Flynn & de Mayo (1951) was used, with 36 × 5 cm. strips of Whatman no. 1 paper and phosphate buffer of pH 8·0, $\mu = 0{\cdot}1$. Voltage applied 126 v. and time of running 11 hr.

Spot tests. Aldehydes were detected by the methods of Davidson & Gregory (1932) and Bland (1949); phenolic groups according to Davidson & Gregory (1932) and by spraying chromatograms with diazotized *p*-chloraniline (2 vol. of 0·02 M-*p*-chloraniline in 0·25 N-HCl + 1 vol. of 0·5 % $NaNO_2$).

Ultraviolet absorption spectra. These were determined using a Unicam Spectrophotometer with a 2 mm. cell and water as the blank.

RESULTS

Chromatography of the fluorescent materials

In order to examine for the possible presence of fluorescent materials in normal hair a sample of uninfected hair from children was extracted in the same way as for infected hair. The amount of hair used was approximately 50 times that of the infected hair and the resultant extract showed considerable fluorescence of a darker blue than that seen in infected hair extracts. A chromatogram developed with *n*-butanol/glacial acetic acid/water mixture showed: (*a*) some faint fluorescence which had not moved from the starting point; (*b*) a dark blue slowly moving fluorescent spot; (*c*) a faint blue fast moving spot.

Extracts of hair infected with any of the three fungi contained considerable amounts of material which reacted with ninhydrin, and an attempt was made to find solvent mixtures which would give good separation of the fluorescent materials and at the same time separation from ninhydrin-reacting substances. The butanol/acetic acid/water and butanol/ethanol/ammonia/water mixtures proved the most satisfactory of the many tried.

Extracts from hair infected by each fungus were examined separately and compared. Chromatograms of all three extracts showed a main brilliant greenish blue fluorescent spot, which was not present in the extract of normal hair, moving in each case and with all solvent mixtures used to an identical position (Pl. 1, fig. 1*a–c*; fig. 2*a*, *b*). A dark blue fluorescent spot was also clearly seen a little below this in extracts of hair infected by *Microsporon canis* and *Trichophyton schoenleini* (Pl. 1, fig. 1*d–f*; fig. 2*c*) but was only very faintly visible in chromatograms of the *Microsporon audouini* extracts. This latter blue spot occurred in the same position as the slow moving dark blue spot present in the chromatogram from normal hair. A faint light-blue fluorescent spot was present in extracts from hair infected by *M. canis* and showed much more strongly in that from the *Trichophyton schoenleini* infection (Pl. 1, fig. 1*g–i*). This spot was not found in normal hair extract and has not been seen in extracts from hair infected by *Microsporon audouini*. Additional fluorescent spots, yellow and light blue, were present only in the extract from *Trichophyton schoenleini* infected hair (Pl. 1, fig. 1*j–m*).

Pl. 1, fig. 2, shows the result of spraying with ninhydrin a chromatogram prepared from the extracts of *Microsporon canis* and *M. audouini* infected hair and indicates the considerable separation which can be achieved of the fluorescent materials from ninhydrin-reacting material. This fact was utilized in the purification of the main green fluorescent material from such extracts. The initial hair extract was run on a chromatogram on Whatman no. 3 paper with the *n*-butanol/acetic acid/water mixture, the main fluorescent band eluted with 1·17 N-NH_3 by shaking at room temperature for 20 min. followed by immediate concentration *in vacuo* to low volume and the procedure repeated using the *n*-butanol/ethanol/ammonia/water mixture. The final eluate was not entirely free from ninhydrin-reacting material.

Attempts to purify the material by column chromatography using kieselguhr, Decalso and Whatman powdered filter-paper showed no adsorption of the fluorescent material. When the fluorescent material was dissolved in 60 % (v/v) *iso*propanol and added to a column of alumina (British Drug Houses Ltd. alumina for chromatographic analysis) the fluorescent substance was strongly adsorbed at the top of the column and resisted all attempts at elution. Mixtures of kieselguhr and alumina in differing proportions yielded the same result.

Paper electrophoresis. Further evidence for the identity of the green and dark-blue fluorescent spots in the extracts from *Microsporon canis* and *Trichophyton schoenleini* infected hair was obtained by paper electrophoresis (Pl. 2, fig. 3*d–g*), but the light-blue fluorescent spot seen in chromatograms of these extracts was only seen in the extract from the *T. schoenleini* infection (Pl. 2, fig. 3*b*). All three materials moved towards the anode indicating that they were negatively charged at pH 8·0.

The additional fluorescent materials found in chromatograms from the *T. schoenleini* infection are also seen. (Pl. 2, fig. 3*a*, *c*); the blue fluorescent spot moved a short distance towards the anode, while the yellow spot moved a very short distance from the starting-point towards the cathode, this movement being almost certainly due to the electro-endosmosis occurring during the experiment.

Ultraviolet absorption spectra. The spectra of a number of the purified extracts from *Microsporon canis* and *M. audouini* infected hair were examined in the range of pH 6–7. All showed strong end absorption but no constant characteristic curve was obtained, as also reported by Robinson *et al.* (1953) for extracts of *M. audouini* infected hair. Below 300 mμ the results varied considerably from one extract to another; with extracts from smaller amounts of fluorescent hair no specific absorption between 300 and 400 mμ. was found. With the more concentrated extracts there was a small amount of light absorption between 310 and 340 mμ. not seen in weaker solutions, although these were still strongly fluorescent. It seems probable that the fluorescent substance has not yet been obtained sufficiently concentrated to give a characteristic spectrum.

Chemical properties

Extracts of the principal greenish blue fluorescent material from *Microsporon canis* and *M. audouini* infected hair, purified by chromatography as described above and concentrated finally to a volume of *c.* 1·0 ml., were subjected to a number of spot tests. The final concentrate was pale yellow in colour, slightly opalescent, exhibited a vivid greenish blue fluorescence in ultraviolet light, and on evaporation to dryness left a just discernible yellowish residue. The tests for aldehyde and phenolic groups used by Davidson & Gregory (1932) both yielded positive results. A sample, 0·1 ml., of the concentrate was dried drop by drop on filter-paper and sprayed with 2:4-dinitrophenylhydrazine but no colour change was discernible. Treatment of 0·2 ml. of concentrate with this reagent and warming for 30 min. at 37° led to the separation of a few crystals, visible microscopically, but insufficient for characterization. The presence of a phenolic substance was confirmed by treatment with diazotized *p*-chloraniline. The aldehyde and phenolic group tests on the crude extract from *Trichophyton schoenleini* infected hair were also positive. Spraying of chromatograms with diazotized *p*-chloraniline followed by exposure to ammonia vapour, however, showed the phenolic substance to be associated with the dark blue fluorescent spot and not with the main fluorescent material. This finding was confirmed by spraying the results of paper electrophoresis with the same reagent; no colour with the phenolic reagent was given by the main fluorescent material. The presence of this dark blue fluorescent substance in the final eluate from chromatography was due to incomplete separation of the two fluorescent materials on the Whatman no. 3 paper. Examination of the crude extract of the *T. schoenleini* infected hair and of chromatograms developed from it gave the same results.

The fluorescence of extracts from hair infected with any of the three fungi was quenched immediately by the addition of bromine water or by sodium hydrosulphite, being regenerated in the latter case by shaking the solution in air, thus confirming the finding of Robinson *et al.* (1953) with a fluorescent extract of *Microsporon audouini* infected hair. There was no change in the fluorescence on treatment with hydrogen peroxide or sodium sulphite. No colour was given with ferric chloride.

DISCUSSION

Whilst growing as parasites in the hair root, *Microsporon canis*, *M. audouini* and *Trichophyton schoenleini* all cause the production of fluorescent substances in the cortex of the hair. They do not, however, produce these substances in artificial culture, so that the supply of fluorescent material is necessarily very limited. A number of attempts have been made to infect animals experimentally with *Microsporon canis*, but only small areas of infection have been obtained in guinea-pigs and all attempts to infect rats and mice have failed. The initial extract from the infected hair is a complex mixture of breakdown products of keratin and, presumably, other metabolic products from the invading fungus. Separation from ninhydrin-reacting substances by the techniques described was not complete and it is still an open question as to whether the main fluorescent substance is a peptide; Davidson & Gregory (1932) reported no ninhydrin-reacting material in their extracts, while Robinson *et al.* (1953) concluded from infra-red analysis of their extract from *M. audouini* infected hair, that the material resembled a peptide. They produced, however, no evidence that their procedure for purification had separated the fluorescent substance from all the other materials and in particular from the products of keratin breakdown known to be present in the initial aqueous extracts of infected hair, or from the fluorescent substances now shown to be present in normal hair. In view of the small amount of material available chemical properties can only be examined by spot tests, and every endeavour has been made to relate these results to areas of fluorescence as shown by paper chromatography, and only results which have been consistently positive on a series of extracts over the past 4 years have been recorded.

We wish to thank Dr J. T. Ingram for permission to obtain infected hair from his patients, Dr J. Walker and Dr C. J. La Touche for identifying cultures from many of the patients, Mr T. J. Bowen for advice on paper electrophoresis techniques and Mr C. N. England for carrying out the photography.

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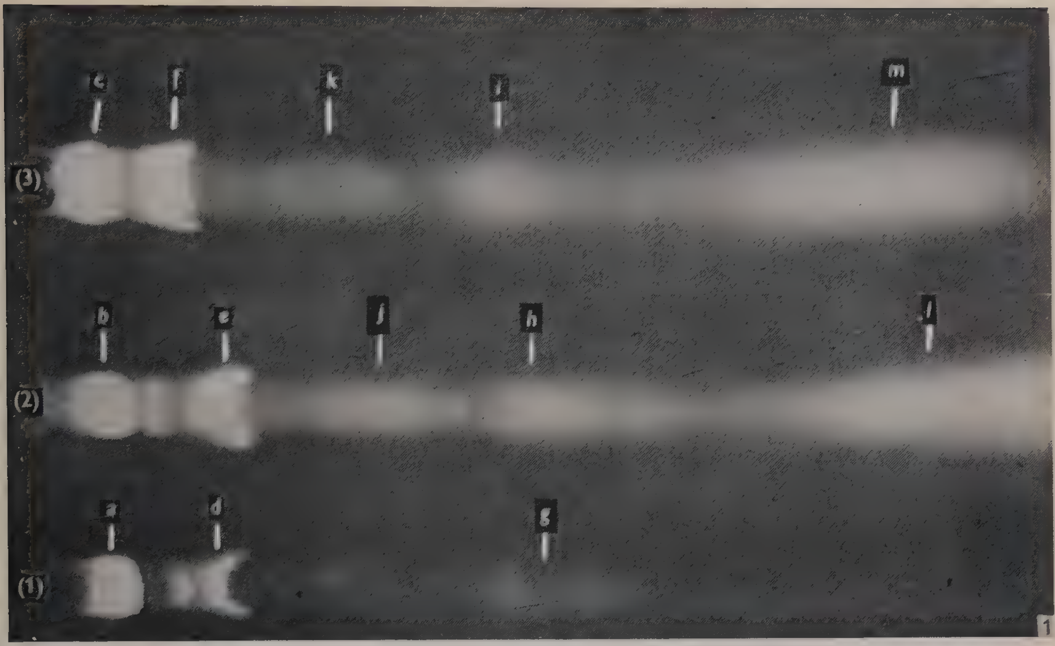
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Fig. 1

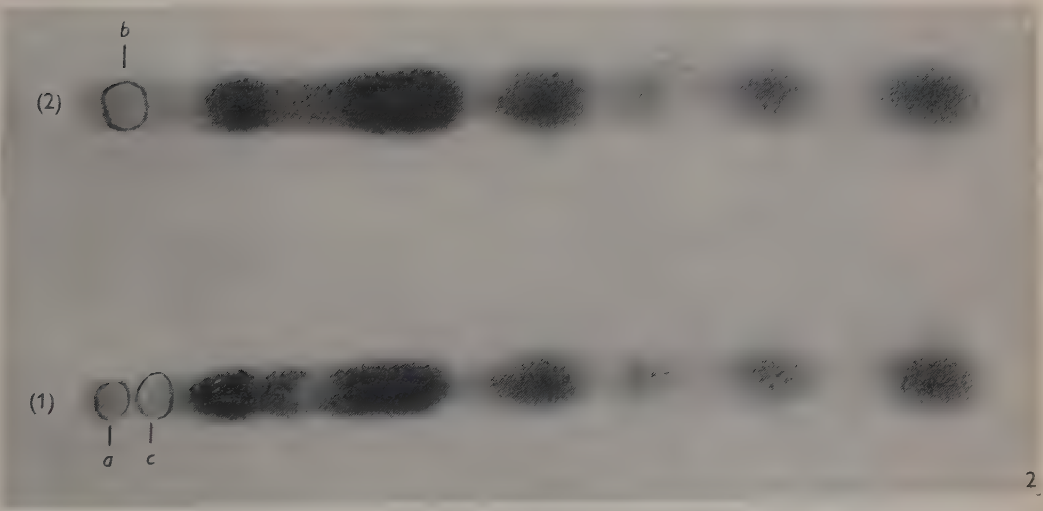
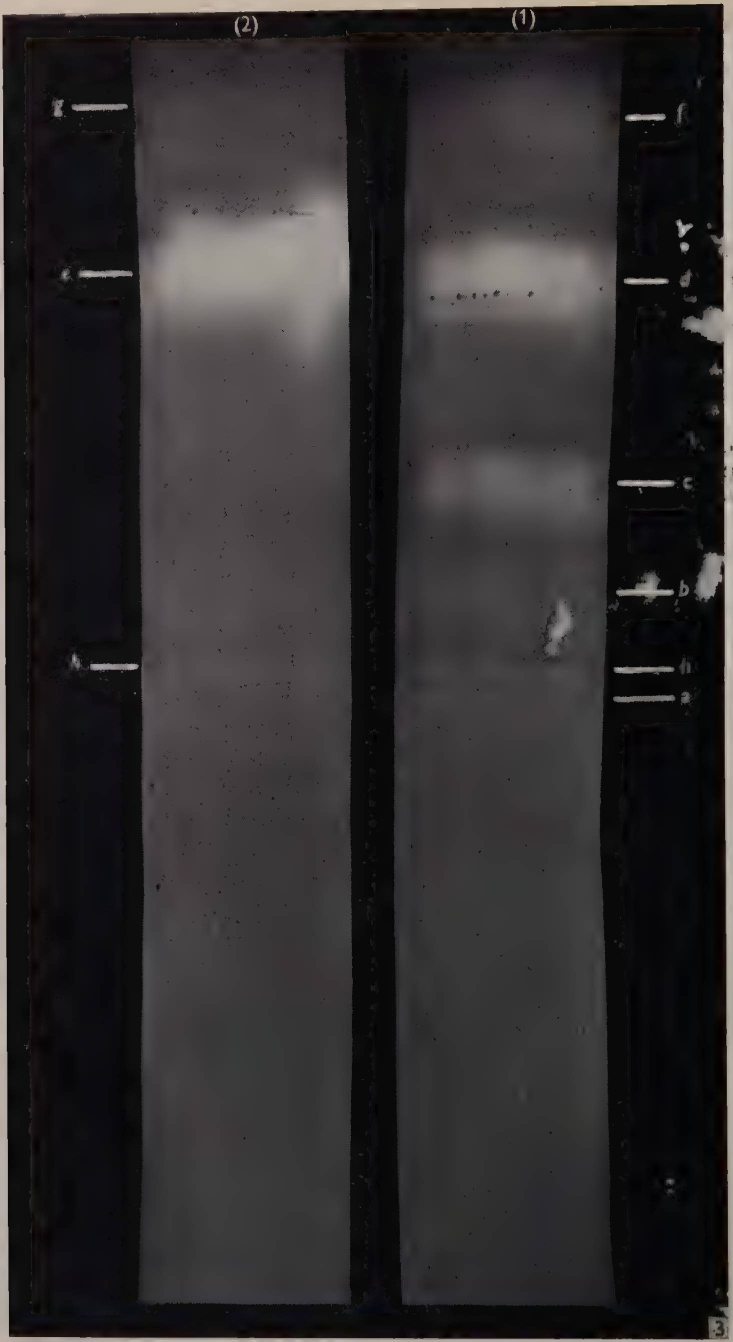


Fig. 2

F. W. CHATTAWAY AND A. J. E. BARLOW—FLUORESCENCE OF DERMATOPHYTES *IN VIVO*. PLATE 1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F. W. CHATTAWAY AND A. J. E. BARLOW—FLUORESCENCE OF DERMATOPHYTES *IN VIVO*. PLATE 2

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Chromatogram developed with *n*-butanol/glacial acetic acid/water on Whatman no. 3 paper of crude extracts of hair infected with (1) *Microsporon canis*, (2) *Trichophyton schoenleini*, (3) a mixture of extracts (1) and (2). Areas of fluorescence: (*a*), (*b*), (*c*) brilliant greenish blue; (*d*), (*e*), (*f*), dark blue also seen in extracts of normal hair; (*g*), (*h*), (*i*) light blue; (*j*), (*k*) yellow, (*l*), (*m*) light blue. Photographed in ultraviolet light using Ilford S.R. panchromatic plate with an Osira black lamp, MBW/V, at a distance of 1 ft. with exposure time 10 sec. at f. 16.

Fig. 2. Chromatogram developed with *n*-butanol/ethanol/ammonia solution sp.gr. 0·880/water on Whatman no. 3 paper of crude extracts of hair infected with (1) *Microsporon canis*, (2) *M. audouini*. Spots (*a*) and (*b*) are areas of brilliant greenish blue fluorescence; spot (*c*) an area of dark blue fluorescence, also seen in extracts of normal hair. Dark areas represent colours produced on spraying with 1% ninhydrin in *n*-butanol.

Plate 2

Fig. 3. Paper electrophoresis of crude extracts of hair infected with (1) *Trichophyton schoenleini*, (2) *Microsporon canis*. Areas of fluorescence: (*a*) yellow; (*b*), (*c*) light blue; (*d*), (*e*) bright greenish blue; (*f*), (*g*) dark blue. Spot (*h*) was the starting-point. Photographed in ultraviolet light using Ilford S.R. panchromatic plate with two Osira black lamps at a distance of 1 ft. with exposure time 16 sec. at f. 8.

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Leifson, E. & Hugh, R. (1954). *J. gen. Microbiol.* **11**, 512–513.

Alcaligenes denitrificans n.sp.

By E. LEIFSON and R. HUGH

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SUMMARY: A new species of the genus *Alcaligenes* is described which is able to produce gas from nitrate.

The ability to reduce nitrate with formation of nitrogen gas is common to several kinds of bacteria, particularly the non-fermentative Gram-negative rods. The named bacteria of this type about which we have personal knowledge are *Pseudomonas aeruginosa, P. stutzeri* and *Malleomyces pseudomallei*. Various strains of *Brucella* of all species may produce a small amount of gas from nitrate. The phenomenon appears to be most common among the polar flagellated organisms of the *Pseudomonas* genus.

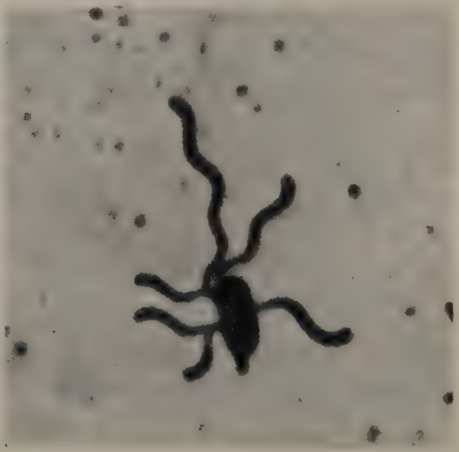

Fig. 1. *Alcaligenes denitrificans* n.sp. showing typical flagellation. Leifson flagella stain (Leifson, 1951). Photomicrograph, ×3600.

Among some 100 strains of peritrichously flagellated Gram-negative rods in our collection are eight which produce gas from nitrate. Six of these are old stock cultures from various sources; all oxidize glucose and some xylose. The other two strains were received from L. R. Fredrich (Purdue University) and were isolated from soil. These two strains appear to be alike and do not attack any of the carbohydrates tested. They appear to be typical of the *Alcaligenes* genus. We suggest that they be recognized as a species of *Alcaligenes* with the name *Alcaligenes denitrificans* n.sp.

DESCRIPTION

Morphology. Rod shaped, 0·5 by 1·0 μ, ends rounded, usually occurring singly or in short chains. Motile. Gram-negative. Flagellation peritrichous with 3 to 10 flagella/organism. Flagella all of one type. Measurement of 20 flagella gave a mean flagellar wavelength (spiral period) of $2{\cdot}46 \pm 0{\cdot}044\,\mu$., and a mean flagellar amplitude (spiral width) of $0{\cdot}49 \pm 0{\cdot}024\,\mu$. In Fig. 1 is reproduced a photomicrograph showing the flagellation of the organism.

Cultural characteristics. Colonies on agar smooth, edges entire, semi-translucent. Growth on agar slants abundant, smooth, colourless and semi-translucent. Growth in agar stab confined mainly to or near the surface. Growth in broth uniformly turbid, moderate pellicle formation. No growth in nutrient broth under anaerobic conditions. Temperature relations, mesophilic; pH relations, neutrophilic; oxygen relations, aerobic; pigmentation, none.

Physiological characteristics. Carbohydrates not attacked include lactose, sucrose, glucose, maltose, mannitol, xylose, arabinose; urea not hydrolyzed; gelatin not liquefied; H_2S not produced; citrate utilized in Simmons citrate agar; nitrate reduced with formation of nitrite and/or nitrogen gas; litmus milk—turns blue.

Habitat. Isolated from soil.

Type strain. A culture has been deposited in the National Collection of Type Cultures, London (NCTC 8582).

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Eighteenth General Meeting in the Institution of Electrical Engineers, Savoy Place, London, on Thursday, Friday and Saturday, 8, 9 *and* 10 *April* 1954. *The following communications were made:*

COMMUNICATIONS

SYMPOSIUM ON AUTOTROPHIC MICRO-ORGANISMS

The No-man's Land between the Autotrophic and Heterotrophic Ways of Life. By D. D. WOODS and JUNE LASCELLES (*Department of Biochemistry, University of Oxford*)

The Nature and Relationships of Autotrophic Bacteria. By K. A. BISSET and JOYCE B. GRACE (*Department of Bacteriology, University of Birmingham*)

Some Aspects of the Physiology of Thiobacilli. By K. BAALSRUD (*Biologisk Laboratorium, Oslo*)

Some Aspects of the Physiology of the Nitrifying Bacteria. By JANE MEIKLEJOHN (*Rothamsted Experimental Station, Harpenden*)

The Biochemistry of the Nitrifying Bacteria. By H. LEES (*Department of Biological Chemistry, University of Aberdeen*)

The Nitrogen Metabolism of the Blue-green Algae (Myxophyceae). By G. E. FOGG and MIRIAM WOLFE (*Department of Botany, University College, London*)

Ammonia and Nitrate Assimilation by Green Algae (Chlorophyceae). By P. J. SYRETT (*Department of Botany, University College, London*)

Mechanism of Photosynthesis. By H. GAFFRON (*Institute of Radiobiology and Biophysics, University of Chicago*)

The Photolitho-autotrophic Bacteria and their Energy Relations. By H. LARSEN (*Department of Chemistry, Norges Tekniske Högskole, Trondheim, Norway*)

The Utilization of Organic Compounds by Photosynthetic Bacteria. By S. R. ELSDEN (*Department of Microbiology, University of Sheffield*)

The Role of Accessory Pigments in Photosynthesis. By L. R. BLINKS (*Hopkins Marine Station, Pacific Grove, California*)

Problems in the Mass Cultivation of Photo-autotrophic Micro-organisms. By E. C. Wassink (*Laboratorium voor Plantenphysiologisch Onderzoek der Landbouwhogeschool, Wageningen*)

The Economic Importance of Autotrophic Micro-organisms. By K. R. Butlin and J. R. Postgate (*Microbiology Section, Chemical Research Laboratory, Teddington*)

ORIGINAL PAPERS

On the Individuality of Micro-organisms. By W. H. Hughes. (*The Wright-Fleming Institute of Microbiology, London*)

Titrations of sensitivity to antibiotics have shown that the cells in cultures from single staphylococci and other organisms vary considerably. The more resistant cells can be selected and populations with a higher average resistance can be obtained (Hughes, 1952; Eagle, Fleischman & Levy, 1952). The results suggest continuous variation rather than clear-cut stepwise mutation, and would be accounted for if it could be shown that when bacteria divide the daughter cells differ from each other.

That the cells obtained from the division of a single cell may at times be different from each other has already been demonstrated (Hughes, 1953) in the case of a strain of *Escherichia coli* in which a small percentage of the cells were seen to divide so that one daughter cell formed a normal colony, while the other continued to elongate but was unable to divide under the anaerobic conditions provided.

If advantage is taken of the action of antibiotics, particularly penicillin, on single cells of *Proteus vulgaris* it can be shown that there are differences between the individual cells in micro-colonies of from two to thirty-two individuals. Four tests can be applied to each cell: (1) ability to survive, (2) to grow, (3) to divide, and (4) to resume growth on transfer to normal media. Using these differences the inheritance in individuals can be studied and a family tree constructed. The classical experiments on continuous variation in plants can be reproduced using bacteria, but starting with a single cell instead of two parents. The size that the colony of *E. coli* Brent reaches in anaerobic culture in a given time varies from cell to cell; if subcultures of the large and small colonies are made populations can be selected which grow on the average faster or slower than the parent and these can be again reselected.

The results closely resemble those obtained for hyaluronidase production in the staphylococcus by Rogers (1953).

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On the Formation of Invertase by *Saccharomyces fragilis* Jorgensen. By A. Davies (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The conditions under which logarithmically growing *Saccharomyces fragilis* synthesizes invertase have been studied using the principle of continuous culture. It has been found that, when grown in a medium containing glucose or sucrose as carbon source, the main factor controlling the formation of invertase is the sugar concentration in the culture. When the glucose concentration is 0·002 % or less, very high values, up to 100 units/mg. dry weight (the unit of activity is the amount of enzyme which hydrolyses 1 mg. of sucrose in 1 hr.) of invertase are observed, while at 0·02 % the activity is reduced to 2 units/mg. dry weight, and at a glucose concentration of 2 % the formation of the enzyme is almost completely suppressed, the activity being as low as 0·03 unit/mg. dry weight. A similar effect is obtained with sucrose, but the inhibition with excess sucrose is not so marked as that with excess glucose.

The mean generation time and the ammonia concentration have little, if any, effect on invertase formation. In both cases any large variation in activity which occurs with a change in mean generation time or in ammonia concentration can be accounted for by simultaneous variations in sugar concentration.

If in a medium containing excess sucrose the mean generation time is long (>6 hr.) the observed invertase activity can account for the disappearance of slightly more sucrose than is actually observed, but at short mean generation times more sucrose disappears from the medium than can be accounted for by the invertase activity. It would seem that there may be other means of utilizing sucrose than hydrolysis by invertase.

An Antagonist of Dihydrostreptomycin produced by *Pseudomonas pyocyanea*. By J. W. Lightbown (*National Institute for Medical Research, Mill Hill, London*)

Pseudomonas pyocyanea produced during logarithmic growth a substance capable of antagonizing the activity of dihydrostreptomycin in very low dilution. The production of antagonist was dependent on vigorous aeration of culture, the antagonist was destroyed by too high an aeration rate or by foaming. A cup plate assay of activity was developed depending on the production of zones of exhibition by antagonist in plates seeded with *Bacillus subtilis*, and containing an inhibitory concentration of dihydrostreptomycin.

The antagonist was produced in chemically-defined media containing ammonium lactate, K_2HPO_4 and $MgSO_47H_2O$. It was extracted from acidified whole culture by a minimum volume of *n*-butanol, the solvent was removed by distillation under nitrogen and the crude extract fractionated by a method depending on varying solubility of different constituents in mixtures of benzene and petroleum ether. A crystalline preparation was obtained with a formula $C_{17-18}H_{23-24}NO_2$, it had been recrystallized to constant activity.

The crystalline material would antagonize 500 to 1000 times its weight of dihydrostreptomycin against *B. subtilis* and *B. pumilus* under appropriate conditions, its activity was decreased by increase in pH. It would antagonize the activity of dihydrostreptomycin against *Staphylococcus aureus*, but to a negligible extent against *Bacterium coli*. The antagonist would not promote growth of the test organism if the latter had been exposed to dihydrostreptomycin at 37° for more than 4 hr. In the absence of dihydrostreptomycin, relatively high concentrations of antagonist exerted an inhibitory effect against *B. subtilis*, and, at pH 7·2 it was 10 times more active than dihydrostreptomycin. The antagonist inhibited growth of *S. aureus* but not *Bact. coli*. At low concentrations, the antagonist caused stimulation of growth in the absence of dihydrostreptomycin, under certain conditions.

Dihydrostreptomycin was much more active as an inhibitor of growth at high aeration rates. It was under conditions of high aeration rates that the antagonist was most effective in antagonizing dihydrostreptomycin.

Observations on the Inhibitory and Growth-promoting Activities of Streptomycin and Dihydrostreptomycin on Bacteria. By F. L. Jackson (*Cowburn Research Fellow, King's College Hospital Medical School, London*) and J. W. Lightbown (*National Institute for Medical Research, London*)

The effects of streptomycin sulphate, dihydrostreptomycin and streptomycin antagonist (Lightbown) on a streptomycin sensitive staphylococcus and a streptomycin dependent variant (supplied by Dr Mary Barber) and on a streptomycin sensitive strain of *Bacillus subtilis* have been investigated.

The antagonist counteracts the inhibition of the sensitive organisms by the streptomycin derivatives used, but above a certain streptomycin level the antagonist is no longer effective. Increased antagonist concentrations, in the absence of streptomycin, are themselves inhibitory, so that the antagonist can itself act as an 'antibiotic'.

The streptomycin dependent variant growing in the presence of streptomycin is inhibited by the antagonist. High concentrations of streptomycin inhibit the dependent strain, and this inhibition too can be counteracted by antagonist. Whereas streptomycin sulphate and dihydrostreptomycin have equal activity as inhibitors of the sensitive parent staphylococcus, streptomycin sulphate is twice as active as dihydrostreptomycin as a growth promoter for the dependent variant. In streptomycin-containing cultures aerated by shaking, inhibition of the dependent strain by antagonist can be counteracted by adding more streptomycin.

In seeded agar, inhibition zones, whether produced by streptomycin against a sensitive organism or by antagonist against a dependent organism, are wider at the surface than in the depths. Exhibition zones, produced by streptomycin against a dependent organism or by antagonist against a streptomycin sensitive organism in the presence of streptomycin, are wider in the depths than at the surface. With the sensitive staphylococcus and with *B. subtilis* exposure to increasing streptomycin concentrations in the medium leads to obligate microaerobic growth, but the effect is reversed with the dependent strain.

Unlike the parent streptomycin sensitive strain of staphylococcus, the dependent strain does not grow anaerobically. The parent strain is more sensitive to streptomycin aerobically than anaerobically, and the antagonist does not counteract the inhibitory effect of streptomycin anaerobically. A variant derived from the dependent strain was found to be about as sensitive to streptomycin as the original parent strain, but in colonial morphology and inability to grow anaerobically it resembles the dependent strain.

The *B. subtilis* used in these experiments, sensitive to streptomycin by the usual criteria, can be stimulated by streptomycin and even made to appear dependent on streptomycin in medium containing inhibitory concentrations of antagonist.

The desirability of a unitary theory of sensitivity and dependence is discussed.

The Formation of Diazotizable Amine and Hypoxanthine by a Yeast. By N. CHAMBERLAIN and C. RAINBOW (*Department of Applied Biochemistry, University of Birmingham*)

The diazotizable amine, which accumulated in cultures of Yeast 47 grown in a defined medium containing added methionine and suboptimal biotin (Chamberlain, Cutts & Rainbow, 1952), differed from 4-amino-5-imidazole-carboxamide and the corresponding amidine, but possessed some biological activity as a substituent for purines. Spectrophotometric measurements indicated that the amine lacked the complete 6-membered ring structure of the purine skeleton. The accumulation of amine in culture filtrates was depressed by the presence of adenine or certain amino acids, aspartate, norleucine, norvaline and threonine being the most effective.

Those cultures which accumulated amine also accumulated hypoxanthine. Since hypoxanthine substituted for adenine and guanine in the growth of a strain of *Lactobacillus brevis* requiring both of the latter purines, it is suggested that hypoxanthine is a bio-precursor of both adenine and guanine. Hypoxanthine may therefore accumulate in cultures of Yeast 47 as a result of a biosynthetic block, conditioned by biotin deficiency, which prevents the conversion of hypoxanthine to adenine and guanine.

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The Synthesis of Methionine by *Streptobacterium plantarum* 10S. By M. J. CROSS (*Department of Biochemistry, University of Oxford*)

The replacement of serine by CO_2 or leucovorin in the presence of glycine for the growth of *Leuconostoc mesenteroides* P.60 has been extensively studied (Lascelles, Cross & Woods, 1954; Cross, 1953). Radioactive tracer experiments show that CO_2 does not contribute to the β-carbon of serine. Other evidence suggests that CO_2 may partially inhibit the inactivation of some co-enzyme derived from folic acid and essential for serine synthesis.

More recently Gibson (unpublished observation) found that the requirement of *Streptobacterium plantarum* 10S for methionine can be satisfied by homocysteine and CO_2—a second example of the apparent replacement of a methyl group by CO_2. In this case leucovorin does not replace CO_2. Again tracer studies show no incorporation of CO_2 into the methyl group.

In parallel experiments the incorporation of formate into the methyl group of methionine by growing cultures of *Sb. plantarum* 10S was examined. Formate was incorporated with a dilution of about 0·5 : 1·0 both in the presence and in the absence of exogenous serine, whilst in neither case was any significant activity located in the serine of hydrolysed cells.

These results would seem to eliminate one possible route for formate incorporation into methionine by this organism based on two paths known to exist in certain bacteria—the synthesis of serine from glycine and formate (Lascelles & Woods, 1950) followed by the synthesis of methionine from serine and homocysteine (Gibson & Woods, 1952). They accord with the observations of Arnstein & Neuberger (1953) with intact rats, and of Berg (1953) with cell-free extracts of pigeon liver.

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Concurrence of Autotrophic and Heterotrophic Metabolism in Growing and in Resting Cells of Sulphate-reducing Bacteria. By J. C. Senez (*Centre National de la Recherche Scientifique, C.R.S.I.M., Marseilles*)

Preliminary growth experiments with pure strains of sulphate-reducing bacteria have shown that, in the presence of both molecular hydrogen and lactate or pyruvate, the sulphates present in the medium are reduced simultaneously by autotrophic and by heterotrophic modes of nutrition. The extent to which either mode predominates depends on the strain studied.

Resting cells harvested from autotrophic medium (cultivated in H_2, with bicarbonate as carbon source) have practically the same rate of hydrogen uptake and sulphate reduction with or without added pyruvate. No inhibition of the rate of H_2 uptake is observed in the presence of the organic compounds tested.

With resting cells grown in heterotrophic conditions (lactate + SO_4'', under nitrogen), the rate of H_2 consumption is much higher in the presence than in the absence of added pyruvate, the $-Q_{H_2}$ being respectively 9·9 and 132·3 mm.^{3}/mg. (dry weight)/hr. Addition of lactate has a similar effect, but after a short lag-phase; vitamin-free casamino-acids increase the rate of H_2 uptake, but yeast extract has no effect.

In the manometric systems containing molecular hydrogen and pyruvate, the rate of CO_2 formation from pyruvate by heterotrophically grown cells is of the same order of magnitude as the rate of H_2 consumption ($+Q_{CO_2}=102$, $-Q_{H_2}=132\cdot 3$).

Preliminary Note on a New Variety of *Azotobacter agile*. By J. Smit (*Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen*)

Since Beijerinck's classical investigation in 1901, describing *Azotobacter chroöcoccum* and *Az. agile*, half a century of research has added little of importance. In fact only Lipman's *Az. vinelandii* has been universally recognized as a new species. *Az. Beijerinckii* in so many respects resembles *Az. chroöcoccum*, that its status as a separate species seems less obvious. *Az. indicum*, according to Derx's investigation, has to be eliminated from this group and has been proposed as the type species of a new genus *Beijerinckia.*

Accordingly, only three or four species remain, three of which are found in soil, whereas *Az. agile* has a distinct aquatic distribution. Whenever its isolation from soils was claimed there is every reason to believe that *Az. vinelandii* was involved, as Kluyver and van Reenen have clearly shown.

When isolating *Az. agile* from strawboard waste waters (a medium very rich in this bacterium) my collaborator Dr Wieringa was astonished to find what seemed to be a new species, resembling *Az. agile* in biochemical properties and colour production but distinguished by abnormally large cells and a very limited mobility. Eisenstark in Oklahoma has also observed the formation of large cells in a strain of *Az. agile* (received from Prof. van Niel) but exclusively when grown on beef extract and soil-extract agar. In Wieringa's strains, however, giant cells prevail on all media where growth occurs, and no instance could be found where their size is reduced to normal dimensions, nor are media found on which our strains of normal *Az. agile* are seen to swell to giant proportions.

Where biochemical properties are quite similar and only the constant formation of giant cells and the very much reduced mobility distinguish these strains from normal *Az. agile*, it seems doubtful whether they deserve the status of a separate species and we prefer to regard them as varieties of *Az. agile*.

Stages in the Development of the Capsular Polysaccharide Structure in a *Bacillus* Species. By J. Tomcsik and S. Guex-Holzer (*Institute for Hygiene and Bacteriology, University of Basel, Switzerland*)

Appropriate antibodies reveal a complex capsular structure of *Bacillus megaterium* and of the related *Bacillus* M in the phase-contrast microscope. D-Glutamic acid polypeptide is evenly distributed in the capsule. A species-specific polysaccharide forms a hitherto unknown capsular structure. Its most characteristic elements consist of transverse septa, separating the cells within the capsule, and of polar condensation.

The initial stages of the capsule production were obtained in the present work with *Bacillus* M by using aerated media.

A series of microphotographs demonstrates that the most active portion of the bacterial surface involved in the production of the capsular polysaccharide, is located at the junction of the cell wall and of the cross wall in the multicellular bacillus. At the initial stage, the cross-wall grows outwards and the cell wall is consequently bulged at this place; the multicellular bacillus is then similar to a bamboo. The further growth of these elements leads to the formation of capsular polysaccharide structure. These morphological observations are correlated with the results of immunochemical analysis inasmuch as the same polysaccharide antibody effects visibility of the capsular transverse septum and of the cell wall. In the later stages of capsule formation, other parts of the cell surface might also play a role in the polysaccharide production.

Contrary to the polysaccharide, the polypeptide production is distributed evenly on the cell surface, with the exception of the cell wall and cross-wall junction. In consequence, the polypeptide may show a narrow striation in the young capsule leaving space free for the location of polysaccharide transverse septum. No polypeptide striation could be observed in agar cultures in the fully developed capsules.

Our observations emphasize the usefulness of antibodies in cytological and immunochemical studies of the bacterial surface.

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Polysaccharides of the Yeast Cell Wall. By D. H. Northcote (*Department of Biochemistry, University of Cambridge*)

Three polysaccharides occur in yeast in relatively large amounts: a soluble glycogen and an insoluble glucan, composed of glucose, and a soluble mannan, composed of mannose. The cell wall, 15 % of the total dry weight of the cell, has been isolated by breakage of the cell in the Mickle disintegrator and consists of 29 % glucan, 31 % mannan, 13 % protein and 8·5 % lipid, no other major component can be detected. Glycogen is not part of the cell-wall structure and appears in a 'small particle' fraction centrifuged from the original supernatant.

Methylation of the glucan and hydrolysis show that the molecule is highly branched with an average chain length of nine glucose radicals, the link between these radicals is 1:3 and the inter-chain link 1:2; the majority of the glycosidic linkages are β (Bell & Northcote, 1950). The molecular weight postulated from a study of the water sorption isothermals of the polysaccharide is high, possibly above 6×10^6 (Northcote, 1953). The glucan constitutes a complete membrane.

Chemical analysis of the glycogen has shown it to be very similar to animal glycogen, but the shape of the molecule calculated from viscosity measurements is not spherical, as is rabbit liver glycogen, but elongated.

The mannan is difficult to free from N and is associated with about 0·3 % P even in a 'purified preparation'; this suggests a close association with protein in the original cell wall. Tetramethyl, trimethyl and dimethyl mannose in equal molecular amounts have been isolated by a cellulose column from the hydrolysate of methylated mannan. The trimethyl fraction consists of at least two compounds. This confirms the work of Haworth, Heath & Peat (1941). The mannan is part of a second complete membrane of the cell wall.

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The Action of Lysozyme on the Cell Walls of certain Bacteria. By

M. R. J. Salton (*Department of Biochemistry, University of Cambridge*)

The cell walls of the three lysozyme-sensitive bacteria, *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* are of mucoid nature, possessing polysaccharide and amino-acid containing components. *M. lysodeikticus*, *S. lutea* and *B. megaterium* cell walls contain 44·9, 46·5 and 47·6 % reducing substances (expressed as glucose) respectively, with hexosamine contents of 16·1, 16·3 and 18·0 %. The reducing substances of the walls of all three bacteria have been identified as glucose and hexosamine.

On incubation of the isolated walls in phosphate or ammonium acetate buffer (pH 6·3) with crystalline, egg-white lysozyme, the turbid cell-wall suspensions undergo rapid clearing and no residual structures can be detected in the electron microscope. Dissolution of the walls is accompanied by a liberation of reducing substances and maximum values were attained on incubation of the walls (15 mg. dry weight cell wall/ml., 0·1 M-ammonium acetate buffer, pH 6·3; 50 μg. lysozyme/ml.) for 24 hr. at 37°. Liberated reducing substances (as glucose) amounted to 10·7, 3·4 and 6·7 % of the dry weight of cell wall of *M. lysodeikticus*, *S. lutea* and *B. megaterium* respectively. Examination of cell-wall digests of the three bacteria in the ultra-centrifuge showed that the depolymerized walls behaved as polydispersed material, sedimentation data revealing that the major components of the digested walls of all three bacteria possess molecular weights of the order of 10,000–20,000. Preliminary observations on the electrophoretic behaviour of the non-dialysable fraction of lysozyme-digested walls of *M. lysodeikticus* has shown the presence of several components.

M. lysodeikticus cell-wall digests give a strong Morgan & Elson reaction for acetylhexosamine and the absorption spectrum of the colour complex is identical to that given by acetylglucosamine. Chromatography of the cell-wall digest and the dialysable material from lysozyme-treated *M. lysodeikticus* walls showed material reacting with acetylhexosamine reagents, but its behaviour is not identical with that of acetylglucosamine. The absence of free glucose in *M. lysodeikticus* digests was demonstrated by paper chromatography and by means of glucose oxidase.

A 'Dextran-like' Polyglucose formed by a *Lactobacillus* Species. By NANCY F. MILLIS (*Research Station, Long Ashton, Bristol*), I. HUSAIN, A. N. HALL and T. K. WALKER (*College of Technology, University of Manchester*)

One of us (N.F.M.) isolated from cider a rod-shaped, lactic acid-forming micro-organism, which possessed some characters similar to those of *Lactobacillus pastorianus* Van Laer and in other features resembled *L. brevis* Orla-Jensen. The most notable behaviour of the new organism was its ability to produce viscosity in media containing (*a*) apple juice-yeast extract, (*b*) green-malt extract, or (*c*) casein double digest-yeast extract (CDDYE) with addition of glucose, fructose or especially maltose. In the CDDYE medium in the presence of arabinose or of xylose much acid was formed but little viscosity developed. In synthetic media containing amino acids, simple vitamins and purines, growth did not occur. In CDDYE medium containing maltose, and with nitrogen at the level of 1 g./l., addition of sodium acetate (2 %, w/v) stimulated growth, formation of acid and onset of the viscous condition, but at a higher level of nitrogen (2 g./l.) acetate was inhibitory.

The organism was grown in 3 l. of a yeast extract-peptone extract medium containing maltose (2 %, w/v) for 14 days at 25° in an atmosphere of carbon dioxide. The liquid was passed through a Seitz filter and 9 l. of ethanol were added to the filtrate. The material which separated was several times redissolved and reprecipitated with ethanol, yielding finally 75 mg. of a polysaccharide which, in simple chemical, physical and serological tests, exhibited behaviour similar to that of a dextran. $[\alpha]_D^{20} + 220° \pm 2°$ in 2N-NaOH (*c*, 0·5); mol. wt. 7000. Complete hydrolysis of the polysaccharide yielded solely glucose. Evidence was obtained that a second product causing viscosity in the medium is formed by this organism. This second product is thermolabile and is acidic in character but it does not appear to be a nucleic acid.

The Mechanism of Transport of Phosphate across the Osmotic Barrier of *Staphylococcus aureus*. By P. MITCHELL (*Department of Biochemistry, University of Cambridge*)

This research was planned with three aims in view: by permeability studies to demonstrate the osmotic barrier; by observing the specificity and kinetics of the transport process to formulate a general description of the mechanism; and by examining the chemical and enzymic composition of the osmotic barrier to provide a molecular explanation.

Permeability measurements on *Staphylococcus aureus* (strain Duncan) show that an osmotic barrier exists near the cell surface. In resting cells, although free phosphate ions cannot diffuse across the barrier, a strictly reciprocal exchange of inorganic phosphate can occur across it. The specificity and kinetics of the exchange suggest that the phosphate is carried as R-phosphate groups, moved to and fro across the barrier by thermal agitation—the temperature characteristics of this motion resembling those of reversible protein denaturation. The exchange of the phosphate of the R-phosphate groups in the barrier with inorganic phosphate on either side appears to be enzymically catalysed, the free energy of the R-phosphate bond being conserved.

During glucose fermentation, the influx of phosphate is the same as during rest, but the outflux falls so that the cells accumulate phosphate. An acceptor presumably removes phosphate from the carrier on the inside of the barrier and the carrier is rephosphorylated on the outside by an unknown exchange reaction.

The material of the osmotic barrier can be obtained as small lipoprotein particles after mechanical disintegration of the cells. These particles contain more than 90 % of the total activity of an acid phosphatase which acts outside the osmotic barrier of intact cells. They also contain the cytochrome system and many other enzyme activities. The R-group which carries the phosphate across the osmotic barrier may be the active centre or prosthetic group of the enzyme controlling the phosphate transport and exchange.

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